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Mass overloading in the flow field-flow fractionation channel studied by the behaviour of the ultra-large wheat protein glutenin

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Abstract

Flow field-flow fractionation (FFF) has previously been used in successful fractionation and characterisation of the ultra-large wheat protein glutenin. The many parameters, which may influence the retention behaviour, especially when analysing extremely high-molecular-mass samples such as glutenin, are here reported. Size determination from the sample retention time, using FFF theory, will as a result have a very low accuracy. The need for direct molecular mass determination, such as by light scattering, in combination with FFF, in order to do accurate size measurements of glutenin is pointed out as well as the importance to minimise the overloading.

Keywords: Mass overloading; Flow field-flow fractionation; Light scattering; Field-flow fractionation; Glutenin

1. Introduction

Field-flow fractionation (FFF) is a whole family of different chromatographic-like separation techniques. The separation is obtained along the axis of a thin, open flow channel. Unlike chromatography the retention is caused by an externally applied field, perpendicular to the flow, which concentrates the sample against one of the channel walls, called the accumulation wall. The velocity of migration of a sample along the flow axis is controlled by the steady-state distribution of the sample in the channel. In flow (Fl) FFF [1–3] this distribution is affected by the molecular diffusion coefficient of the sample, D, in addition to the applied volumetric cross flow-rate, F_c, perpendicular across the channel. The thickness, l, of the exponential sample distribution in the channel, is then given by the ratio $D/|U_0|$, where $|U_0|$ is the cross flow velocity at the accumulation wall. The thickness may preferably be expressed in terms of a dimensionless parameter, λ, which is l divided by the channel thickness, w.

The sample retention is characterised by the retention ratio, R, which is defined as $R=t^0/t_R$, where t_R is the retention time and t^0 is the void time. The retention ratio can be approximately related to the dimensionless parameter λ as $R=6\lambda$, which is valid within 2% when $R<0.06$ and 5% when $R<0.15$ [4,5], i.e., $t_R \geq 17t^0$ or $7t^0$, respectively. As long as this approximation is acceptable the sample diffusion coefficient can be directly calculated from

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the retention time, when knowing the channel dimensions and the cross flow-rate. The hydrodynamic radius, r_H, of the sample is then calculated from the diffusion coefficient using the Stokes–Einstein equation. Moreover the molecular mass can be estimated from the diffusion coefficient with some assumptions regarding the molecular shape, as described in previous FFF papers on glutenin characterisation [6,7]. All these relationships are only accurate using an ideal, linear FFF operation where each molecule acts independently of one another. For small-sized samples (M_r<200,000) this is easily obtained due to the diluted concentration of sample material in the channel. For polymeric samples of higher molecular masses, which are particularly subjected to chain entanglement and other interactive processes, the onset of a nonlinear operation, i.e., overloading, may occur at modest concentrations [8,9]. This is due to the build-up of sample material near the accumulation wall, which increases the concentration and endangers the linearity.

Since the first recognition of the overloading phenomena in flow FFF [10] an important experimental requirement of practical FFF operation has been to find conditions that provide an adequate detector signal while still avoiding observable overloading. Since the separation of high-molecular-mass polymers or proteins is especially exposed to overloading, only a small amount of these samples should be injected, which could become a problem for the detection. The FFF separation takes place in the very lowest part of the channel (~1% of w), close to the accumulation wall as shown in Fig. 1, and the sample zone is diluted with solvent from all above streamlines of the parabolic flow profile as it leaves the channel. The sample concentration in the detector cell will therefore be much lower than that in the sample zone in the channel. The use of a frit inlet/frit outlet symmetrical flow FFF channel [11] counteracts this dilution to some extent but probably not much more than the use of an asymmetrical flow FFF channel, which previously has been shown to cause less dilution than the symmetrical version [12].

A number of relevant transport coefficients in the FFF separation are strongly affected by a change of the sample concentration in the channel. In order to understand the cause of overloading, and how to prevent or minimise the phenomena, these effects

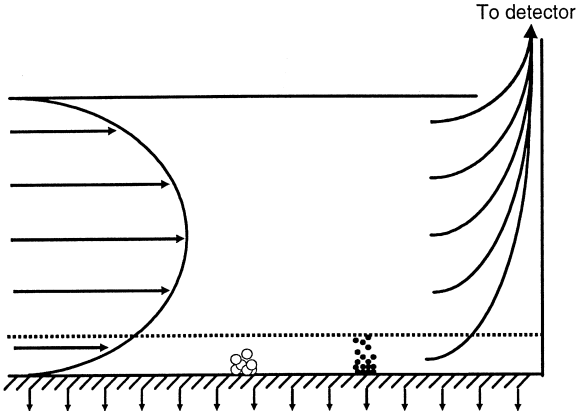

Fig. 1. The separation of sample components in the AsFlFFF channel. Only the very lowest part, close to the accumulation wall (below the dashed line), is used for separation. This results in a dilution of the sample as it leaves the channel and mixes with all above flow streams.

have to be taken into consideration. In the case of flow FFF parameters to consider are the viscosity and the diffusion coefficient. An increase in viscosity and decrease in diffusion coefficient with increased sample concentration is largely accepted and experimentally observed for uncharged polymers. In the case of polyelectrolytes extra energy is required to overcome the interaction between ions in the double layers around the polyelectrolyte and the fixed charges on its surface, which increases the viscosity and decreases the diffusion coefficient with decreasing ionic strength [13]. If the polyelectrolyte chains are flexible the situation is even more complicated and the viscosity as well as the diffusion coefficient will depend on the configuration of the polymer chains. The sample concentration effects for polyelectrolytes are therefore often opposite to those for uncharged polymers. In the absence of electrolyte or at low ionic strength the viscosity increases and the diffusion coefficient decreases with decreasing sample concentration since the concentration of macro-ions as well as their counter-ions decreases, hence reducing the ionic strength of the solvent. These observations have previously been reported in FFF studies on polyelectrolytes at low ionic strength [14,15]. Another parameter to consider is the cross flow-rate, which will be locally reduced by a high zonal sample concentration. The effect of overloading on the retention behaviour of the sample ana-

lysed therefore varies, depending on the parameter most strongly affected and the nature of the sample.

The influence of the carrier composition, more specifically the ionic strength, on the overloading phenomena has previously been pointed out [10,16–19]. For charged polymers it has been shown that a low ionic strength of the carrier solution could completely disturb the retention mechanism by overloading, resulting in early-eluted and sometimes deformed peaks [18,20]. Electrostatic repulsion effects have been identified as a cause of these perturbations to the ideal retention volume [16,17]. Various charge groups of the same sign will repel one another. If the conformation of a polyelectrolyte can change in such away as to increase the distance between the charged groups this occurs in order to decrease the electrostatic energy of the macro-ion. The magnitude of this decrease is reduced as the ionic strength is increased due to the shielding of the charged groups. The addition of salt also reduces the solvation power of the solvent, which makes the polymer–solvent interactions less favourable. This induces a reduction of the volume of the macromolecule to an extent that depends on the polymer properties. The sample layer of charged molecules in aqueous solution will also, as the amount of solute increases, become thicker and the molecules move with faster streamlines due to repulsions, which results in shorter retention times [10,19].

The absence of a stationary phase in FFF makes it especially suitable for the separation and characterisation of ultra large macromolecules. FFF has been shown to be very promising in the separation and characterisation of the ultra-large wheat protein glutenin [6,7,11,21,22]. An accurate size estimation of these glutenin molecules, in terms of molecular mass or radius, is of highest interest due to the correlation of bread making quality with the enormous size of these wheat glutenin polymers [23–25]. The lack of reliable techniques for molecular mass determinations of ultra large molecules has for long limited the possibility to characterise the glutenin [26]. FFF therefore has a great potential of becoming the approach to use in this characterisation. If the FFF retention data are to be used for accurate characterisation of the sample molecular mass or diffusivity however linear operation conditions have to be found, i.e., conditions, which give results that remain invariant even if the experimental conditions should change.

In this study the many experimental conditions, which may have an impact on the retention behaviour of glutenin in the flow FFF channel, have been studied. A variety of sample loads, inlet flowrates, retention ratios, injection procedures and carrier compositions have been used. The influence of each parameter on the retention behaviour has then been determined from the retention time and the calculated hydrodynamic diameter of glutenin. With this procedure the easy onset of nonlinear operation, i.e., overloading of the FFF channel, when analysing these ultra-large wheat proteins will be demonstrated. The low accuracy if, as in previous studies [6,7,22], the retention time is used for size determination of these proteins will be pointed out. Finally the use of a direct molecular mass determination method in combination with the FFF separation technique in order to do accurate molecular mass determinations of macromolecules, such as glutenin, will be encouraged as well as the use of FFF conditions giving linear operation, even though the retention time is not being used for size determination.

2. Theory

Sample overloading in FFF, as in chromatography, refers to a distortion of the elution profile due to a too high sample concentration. This causes various interactions between the sample particles as well as other concentration-dependent effects in the separation channel [9,17]. In FFF a rigorous treatment of the overloading phenomena is further complicated by the distribution of the sample concentration in two dimensions of the channel, the longitudinal and transverse axis. No theory describing overloading in FFF has therefore yet been developed. Some of the most important background equations, which may depart due to overloading, are in this section described in their linear cases.

2.1. Concentration profile

Under the influence of an applied field or gradient, such as the cross flow in flow FFF, the injected

sample migrates towards the accumulation wall, which results in an increased concentration near the wall and a depleted concentration over the remainder of the channel cross section. Since the migration of sample material towards the accumulation wall is balanced by diffusion a steady-state distribution is rapidly established, where the concentration along the transverse x-axis can be expressed by the exponential form [1,27]:

$$c(x,y) = c_0(z)\exp(-x/l) = c_0(z)\exp(-x/\lambda w) \quad (1)$$

where $c_0(z)$ is the sample concentration at the accumulation wall, located at $x=0$.

The highest magnification of the injected sample concentration, with initial concentration c_{inj}, is found at the accumulation wall before any dilution due to band broadening has occurred. The equilibrium wall concentration $c_0(z)$ of such a zone is found by the integration of $c(x,z)$ in Eq. (1) over the full thickness w of the channel $\int_0^w c(x,z)dx = wc_{inj}$ which gives:

$$c_0(z) = \frac{c_{inj}}{\lambda[1-\exp(-1/\lambda)]} \cong \frac{c_{inj}}{\lambda} \quad (2)$$

Since λ commonly falls in the range of 0.005 to 0.05, a substantial gain of concentration c_{inj} prior to band broadening can be expected. Because of this concentration effect, which does not occur in most other separation techniques, overloading is frequently a problem in FFF.

2.2. Retention

The retention parameter λ is for the flow FFF subtechnique expressed as [1]:

$$\lambda_{flow} = \frac{DV^0}{F_c w^2} \quad (3)$$

where V^0 is the void volume of the channel. Under conditions of high retention where the retention ratio, R, can be approximated as $R = 6\lambda$ the diffusion coefficient of the sample can be related to its retention time, t_R, using Eq. (3) in combination with $(t_R/t^0) = 1/R$, as:

$$D = \frac{t^0 F_c w^2}{6 t_R V^0} \quad (4)$$

Eq. (4) can be further used in combination with the Stokes–Einstein equation, $6\pi\eta r_H = kT/D$, to calculate the hydrodynamic radius, r_H, or diameter, d_H, of a sample component from the retention time as:

$$d_H = 2r_H = \frac{2t_R V^0 kT}{t^0 F_c w^2 \pi\eta} \quad (5)$$

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

3. Materials and methods

3.1. Sample material

The glutenin sample was the freeze–dried extracted fraction number 6 of the flour of the wheat cultivar Mexico 8156 (11.7%, w/w, protein) [28,29]. This fraction had been previously prepared, in association with an earlier study [30], by fractional extraction, then adjusted to pH 5.8 by NaOH and freeze–dried for storage. The freeze–fried fraction 6 was either dissolved by gentle stirring for 3 to 9 days at 6 °C or by sonication (Sonifier B-12 with 3 mm diameter microtip probe; Branson Sonic Power, Danbury, CT, USA) at 19–34 W in two to three repeated steps of maximum 15 s [31,32]. As the dissolution buffer a 50 m*M* sodium phosphate buffer, pH 6.8, with 0.25% sodium dodecyl sulfate (SDS) was used. The pH of the buffer was set by mixing 0.988 g of Na_2HPO_4 with 1.000 g of $NaH_2PO_4 \cdot H_2O$. The dissolved mixture was centrifuged at 6000 rpm for 30 min using a WIFUG 102-09 centrifuge (Chemico, Stockholm, Sweden) and the supernatant then placed at 6 °C awaiting analysis.

3.2. Instrumental set-up

The asymmetrical flow field-flow fractionation (AsFlFFF) channel was cut out from a 130 μm plastic spacer and sandwiched between a membrane and a glass wall in a Lucite Plexiglas block having a porous ceramic frit. As the membrane a NADIR UF 10-C regenerated cellulose ultrafiltration membrane (Hoechst, Wiesbaden, Germany), with a molecular mass cut-off of 10 000, was used. The thickness of

the AsFlFFF channel was calibrated to 90 μm using ferritin [33]. The resulting geometrical void volume of the channel was 0.315 ml. An ambient temperature was kept inside the channel at all times.

To the AsFlFFF channel a multiangle light scattering (MALS) instrument (Dawn DSP laser photometer, Wyatt Technology, Santa Barbara, CA, USA), was connected on-line together with an interferometric refractometer [refractive index (RI) detector, Optilab DSP, Wyatt Technology]. Collection and evaluation of MALS and RI data were performed by Astra software (Wyatt Technology). A 0.02-μm filter, Anodisc 25 catalog No. 6809-6002 (Whatman International, Maidstone, UK), made of aluminum oxide with a pore size of 20 nm, was connected after the carrier pump, before the channel, in order to decrease the background noise in the detector. No in-line filter between the channel and the light scattering detector could be used due to the high-molecular-mass components present in the samples.

3.3. AsFlFFF analysis conditions

The impact of different AsFlFFF parameters on the retention behaviour of glutenin in the AsFlFFF channel, were investigated as summarised in Table 1.

3.3.1. Sample volume and injection procedure

The injected sample volume varied between 20 and 500 μl and was injected at a rate of 0.20–0.70 ml/min using a high-performance liquid chromatography (HPLC) pump (Kontron HPLC 422; Kontron Instruments, Milan, Italy). After 0.5–1.0 min of injection the sample was focused another 0.25–0.7 min before being eluted.

3.3.2. Flow-rates

During injection and focusing (Fig. 2A) the inlet flow-rate, F_{in}, to the channel was 2.0 ml/min. It was delivered by a HPLC pump (Kontron HPLC 422; Kontron Instuments) to the channel in both directions, thus focusing the sample in a narrow band after the injection. During the following elution (Fig. 2B) F_{in} in the range of 2.0 to 5.2 ml/min was used. The direction of the inlet flow was then changed to go from the inlet to the outlet, which enabled a division of it into an axial flow through the channel, with flow-rate F_{out}, and a perpendicular cross flow, with flow-rate F_c. The ratio, F_c/F_{out}, set by a restriction at either of the two outlets, varied between 5 and 10. After every sample analysis the channel was washed in the backward direction (Fig. 2C) at 5 ml/min for 3 min. Kontron software (Gynkotek, Germering, Germany) was used to control the two HPLC pumps and the change of flow directions.

Table 1
Range of variation of the parameters studied

Parameter	Range
Sample mass (μg)[a]	7–300
Sample volume (μl)	20–500
Injection + relaxation time (min)	0.75–1.7
Injection volume (μl)	100–700
F_c/F_{out}	5–10
Inlet flow, F_{in} (ml/min)	2.0–5.0
SDS concentration in carrier[b] (%)	0–0.1

[a] Calculated from the RI data of the eluted peaks using a dn/dc value of 0.3 ml/g.

[b] Carrier: 50 mM sodium phosphate buffer, pH 6.8.

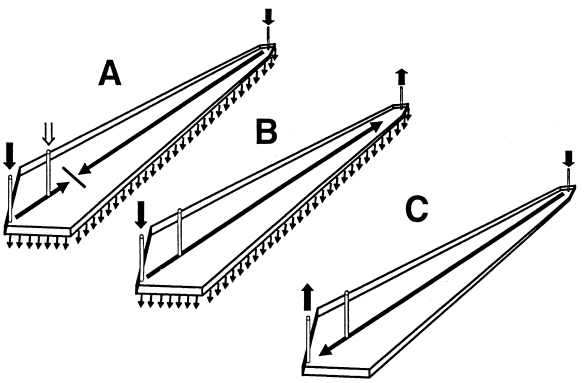

Fig. 2. The three different steps of an AsFlFFF analysis. ↓ = cross flow outlet, ⇓ = sample inlet. (A) Injection and focusing. The inlet flow to the channel is introduced both from the inlet and outlet end thereby concentrating the injected sample in a narrow band at the focusing point where the two flow streams meet. The flow exits through the accumulation wall only, as the cross flow. (B) Elution step. The inlet flow is divided into the axial flow, transporting the sample through the channel, and the perpendicular cross flow that retains the sample components. (C) Washing step. After elution of all sample components the inlet flow is changed to enter the channel in the backward direction from the outlet end, washing any left sample material out of the channel through the inlet end as the cross flow was closed.

3.3.3. Composition of the carrier

As the carrier a 50 m*M* sodium phosphate buffer, pH 6.8, with or without the addition of 0.1% SDS was used. The pH of the buffer was set by mixing 0.988 g of Na_2HPO_4 with 1.000 g of $NaH_2PO_4 \cdot H_2O$. Before use the buffer was filtered through a 0.2 μm regenerated cellulose filter (Sartorius, Goettinger, Germany) and degassed for 15 min in an ultrasonication bath.

3.3.4. Sample load

The impact of the sample load on the retention behaviour of glutenin was investigated in two ways. While keeping the sample volume invariant the sample mass was varied between 7 and 300 μg as described in Table 1. The sample volume was then varied between 20 and 500 μl using a constant sample concentration of 3 μg/μl. As the true injected mass of glutenin could not be precisely known the sample mass was always taken as the mass calculated from the obtained peak areas in the RI fractograms using a refractive index increment, d*n*/d*c*, of 0.3 ml/g for glutenin. The accuracy of these mass values was not further evaluated since it was considered of minor importance. Of greater interest was the relative differences in sample mass and concentration between the different samples, which depend only on the dilution degree of the original sample solution and the volume taken.

4. Results and discussion

A variety of sample loads, injection procedures, inlet flow-rates, cross flow-rates, and carrier compositions were used to investigate the impact of different AsFlFFF parameters on the retention behaviour of glutenin. Each parameter was varied separately and the resulting retention time and measured hydrodynamic diameter of glutenin was then used to determine the influence of the parameter.

4.1. Sample load

Because of the low concentration of the highest-molecular-mass components of dissolved glutenin the sample load on the FFF channel had to be high in the previous FlFFF studies [6,31,32]. When using MALS/RI detection this was especially important in order to get a sufficiently high RI concentration signal to obtain acceptable precision in the molecular mass and size determinations. The weight average molecular mass, M_w, of samples dissolved using gentle stirring and sonication was then determined to $3 \cdot 10^7$ and $5 \cdot 10^6$, respectively [31,32].

4.1.1. Mass load

Using a constant volume of one sample dissolved using gentle stirring and another sample dissolved using sonication, the effect of sample mass on the retention behaviour was investigated by diluting the two samples to a variety of concentrations. As shown in Fig. 3 the measured hydrodynamic diameter at peak maximum, calculated from FFF theory (Eq. (5)), decreased rather dramatically with increasing sample mass. This is a result of the decreased retention time with increasing mass load and is in accordance with previous investigations on polystyrene sulfonates [18], colloidal particles [34] and ferritin [19]. Sample–wall repulsion and repulsive intermolecular interaction (e.g., electrostatic) was there suggested as possible a reason to such a change in the retention behaviour. At low ionic strength ($I = 10^{-3}$–10^{-5} *M*) the intermolecular interactions are mainly repulsive and the electric double layers

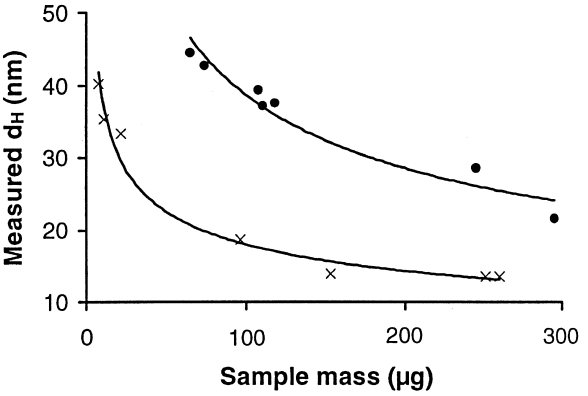

Fig. 3. The hydrodynamic diameter, determined from FFF theory, as a function of the sample mass. The measured hydrodynamic diameter decreased with increasing sample mass due to the decreased retention time of the sample. Both the sample dissolved using gentle stirring (×) and the sample dissolved using sonication (•) showed the same behaviour. Injected sample volume was kept constant at 100 μl.

are large, forcing the sample cloud to become less dense, and therefore displaced further away from the accumulation wall, and be eluted earlier from the channel than expected from FFF theory. The use of an appropriate ionic strength of the carrier could possibly influence the overloading since an increase of the background electrolyte concentration would increase the screening of charged groups and decrease the electric double layers. The net interaction may then instead be attractive. In the case of glutenin the choice of carrier was however also dependent on the insoluble nature of the protein, which required the presence of SDS in it and therefore limited the variability of ionic strength. The effect of the carrier composition on the retention behaviour of glutenin is discussed in Section 4.4.

The same retention behaviour was observed for both the sample dissolved using gentle stirring and the sample dissolved using sonication. The most high molecular mass components ($M_r > 10^7$), only present in the sample dissolved using gentle stirring, caused however a more pronounced overloading. This overloading was so severe that the measured d_H at a similar mass load of the two samples was lower in the sample dissolved using gentle stirring than for that dissolved by sonication, even though the former sample has been proved to have a higher weight average molecular mass [32]. The more pronounced overloading observed for the most high-molecular-mass sample (dissolved using gentle stirring) supports previous observations on polystyrene macromolecules [9], which showed that polymeric samples of higher molecular masses cause an onset of the nonlinear conditions in FFF at lower sample concentrations. The suggested reasons, chain entanglement and other interactive processes, are however not likely in the case of glutenin since that would cause the sample to spend a longer time in the channel. Instead we suggest that the more high-molecular-mass molecules present in the sample dissolved using gentle stirring are affected by a stronger repulsive force, or by a locally reduced cross flow due to their high concentration, that forces them to migrate further away from the accumulation wall than the molecules in the sample dissolved using sonication. As more material could be dissolved when sonication was applied, the concentration signal was high enough for accurate molecular mass determinations. Therefore these samples were not analysed down to the same lowest mass as the sample dissolved using gentle stirring.

As shown in Fig. 4 the overloading also resulted in a change in peak symmetry, causing a tailing peak with a steep front, for the sample dissolved using gentle stirring. The better symmetry for the sample dissolved using sonication once again confirmed that sample components of higher molecular mass, obtained by dissolution of glutenin using gentle stirring, cause a more pronounced overloading.

4.1.2. Volume load

The sample volume ranged between 20 and 500 μl. The sample volume within the analyses of each sample did however not span the complete volume range but only different parts. Only one sample from each dissolution method was used to investigate the effects of sample volume, in order to eliminate the possibility of any other parameter affecting the retention behaviour. As shown in Fig. 5 an increased sample volume gave rise to a similar, but not as drastic, nonlinear behaviour of the glutenin. The less drastic load-dependency by the increased sample volume was also observed in the fractograms (Fig. 6). They showed less peak asymmetry than in Fig. 4, where different sample masses were investigated. These observations are in accordance with the previous results on polystyrene macromolecules [9] and suggest that high-molecular-mass components rather should be injected in a large sample volume of lower

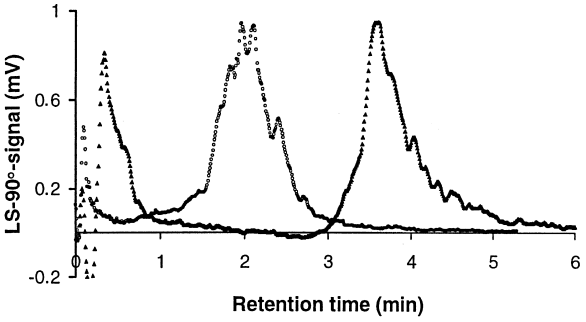

Fig. 4. AsFlFFF fractograms from analyses of glutenin dissolved using: ▲ = Gentle stirring, with sample mass 10 μg, injected in a 100-μl sample volume; ○ = sonication, with sample mass 70 μg, injected in a 100-μl sample volume. F_{in} = 5.0 ml/min, F_c = 4.5 ml/min, F_{out} = 0.5 ml/min, temperature = 24 °C.

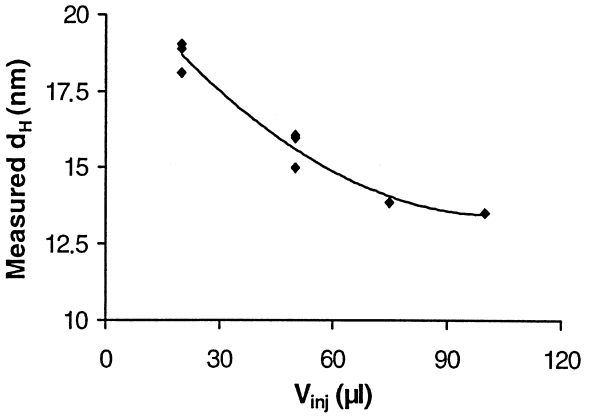

Fig. 5. Hydrodynamic diameter of a sample dissolved using gentle stirring, calculated from the retention times using FFF theory, as a function of the injected sample volume. The sample concentration was kept constant at 3 µg/µl. The measured hydrodynamic diameter decreased as the injection volume increased.

concentration than in a small concentrated sample volume.

4.2. Injection procedure

The glutenin was dissolved in a 0.25% SDS solution but injected onto the FFF channel in a carrier containing 0.1% SDS. Undesirable changes in the conformation, or even precipitation, as the injected solution got in contact with the less SDS concentrated carrier could therefore be possible. The longer time for injection and relaxation, used when larger sample volumes were injected, forced the sample to spend a longer time as a concentrated zone. This increased the risk for sample interactions with the membrane. The time for injection and relaxation was however varied as described in Table 1 without giving any notable changes in the retention behaviour of the sample in the channel or in the size determination of the glutenin (data not shown). None of the above mentioned possible artefacts during injection were experienced as the sample solution and carrier were mixed.

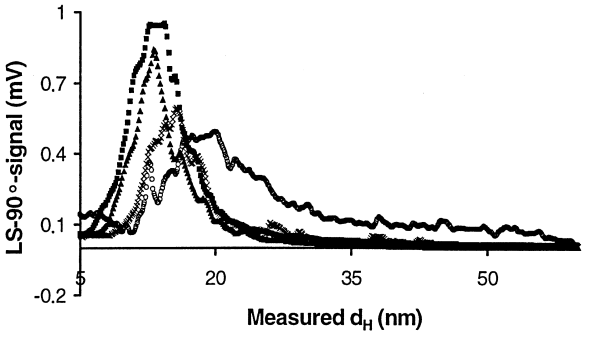

Fig. 6. AsFlFFF fractograms from analyses of glutenin dissolved using gentle stirring at different sample volumes. The sample concentration was kept constant at 3 µg/µl. 20 µl (○), 50 µl (×), 75 µl (▲) and 100 µl (■). F_{in} = 5.2 ml/min, F_c = 4.5 ml/min, F_{out} = 0.7 ml/min, temperature = 24 °C.

4.3. Retention ratio

A primary test of retention in FFF should be one that is designed to show that the experimental retention parameters vary in the expected way with the strength of the lateral ''field''. In flow FFF this is the cross flow-rate. For most field-flow fractionation systems this test can best be achieved in a graphical format by plotting λ vs. the reciprocal of the field strength [10]. A change in F_c should then alter the retention time but leave the measured hydrodynamic size immutable, as described in Eq. (5). An increase in F_c, by increasing the ratio F_c/F_{out} at constant F_{in}, resulted in a longer retention time, as shown in Fig. 7A, but was also, as shown in Fig. 7B, accompanied by an increase in measured hydrodynamic diameter. The decrease in λ, as F_c was increased, was expected to cause the same type of concentration-induced nonlinearities as those at high sample loads. Since high sample loads did not result in an increased retention time, but the opposite, the increase in F_c must have induced a different type of nonlinearity. From Eq. (2) it can be read that the equilibrium wall concentration, $c_0(z)$, increases as the retention parameter, λ, decreases. Since an increased cross flow-rate decreases the corresponding λ any sample component of initial concentration, c_{inj}, would obtain a substantial gain in concentration during the relaxation process before any band broadening occurs. As a result the interaction possibilities between the sample material and the membrane increase. Perturbation from theory when increasing F_c may therefore very well be due to a sample/membrane interaction that increases the sample time in the channel. Such attractive interactions with increasing

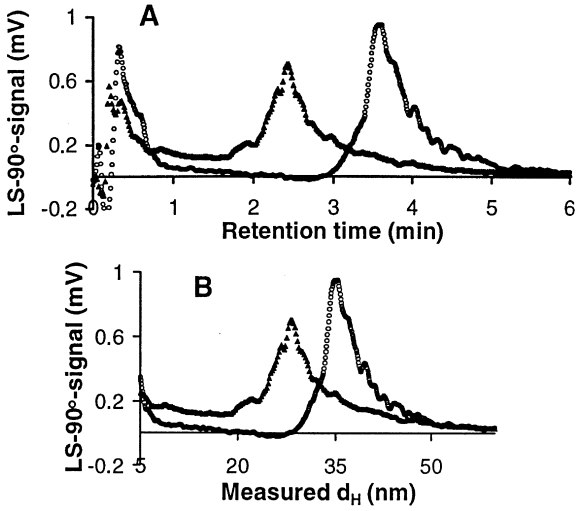

Fig. 7. AsFlFFF fractograms of glutenin dissolved using gentle stirring obtained at different retention ratios, by using different cross flow-rates: (▲) F_c = 4.2 ml/min; (○) F_c = 4.6 ml/min. F_{in} = 5.0 ml/min, temperature = 24 °C. The sample mass was 10 μg injected in a 100-μl sample volume. Detector signal plotted both as a function of retention time (A) and measured hydrodynamic diameter (B).

cross flow-rate have previously been reported in an investigation on fouling on membranes in flow FFF [35]. Any irreversible adsorption of sample material on the membrane could however not be observed in the calculated mass of sample material being eluted from the channel, which remained the same. Due to the unknown concentration of the injected sample an absolute recovery, i.e., the ratio of the sample mass being eluted from the channel to the total injected mass, could not be determined. As judged by the increasing peak area with increasing injection volume in Fig. 6 the elution of different components was however proportional to their levels in the original sample. Fractionation of glutenin and other high-molecular-mass samples is as a result recommended to be performed at low retention levels where moderate compression of the zones keeps nonidealities at a minimum.

At a low retention level (at F_c/F_{out} = 4.5) the impact of the inlet flow-rate, F_{in}, was also investigated. As shown in Fig. 8 the measured hydrodynamic diameter decreased as F_{in} was decreased, which is in accordance with the previous observation on the dependence on F_c/F_{out}. Decreasing F_{in} results

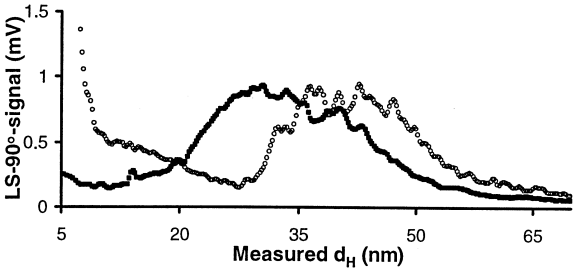

Fig. 8. AsFlFFF fractograms of glutenin dissolved using sonication obtained at different F_{in}: (○) F_{in} = 4 ml/min; (■) F_{in} = 2 ml/min. The F_c/F_{out} ratio was kept invariant at 4.5, temperature = 24 °C. The sample mass was 10 μg injected in a 50-μl sample volume.

in a corresponding decrease in F_c as when F_c/F_{out} was decreased, thereby reducing the risk of adsorption of the sample to the membrane.

4.4. Composition of the carrier

The insoluble nature of glutenin in an aqueous buffer requires a detergent to dissolve the protein. SDS has previously been shown [6] to be a suitable detergent and was therefore used here. In order to investigate the influence of the SDS in the carrier on the retention behaviour of glutenin the sample dissolved by gentle stirring was analysed in both the absence and presence of 0.1% SDS in the carrier. As shown in Fig. 9 the presence of SDS in the carrier had a strong impact on the analysis performance of glutenin. In the absence of SDS the characteristic glutenin peak in the AsFlFFF fractogram disappeared and a very broad, bimodal, peak was obtained. This

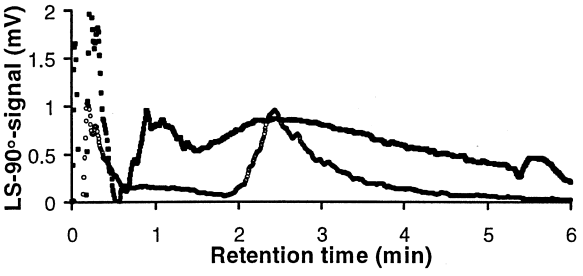

Fig. 9. AsFlFFF fractogram from the analysis of glutenin dissolved using gentle stirring, in the presence (○) and absence (■) of SDS in the carrier. F_{in} = 5.0 ml/min, F_c = 4.2 ml/min, F_{out} = 0.8 ml/min, temperature = 24 °C. The calculated sample mass was 10 μg, injected in a 100-μl sample volume.

seems to be difficult to explain and the reasons for this behaviour could be several.

The often existing charge repulsions within a polyelectrolyte at low ionic strength increases the mean-square end-to-end distance of the molecules and expands the polymer chain [13], which would increase the hydrodynamic diameter and explain the prolonged retention time in the absence of SDS in the carrier. These electrostatic interactions are reduced by the addition of salt, which shields the charge groups from each other and the expansion is therefore reduced as SDS is added [36]. On the other hand, the addition of an electrolyte to a polymer solution can reduce the solvating power, which makes polymer–solvent interactions less favourable than in the absence of electrolyte. This induces a reduction of the volume of the polymer molecule, to an extent that is dependent on the properties of the polymer, an effect that is magnified for polyelectrolytes [37]. This could explain the early elution. Moreover, electrostatic repulsion can also occur between the sample molecules themselves and between the sample molecule and the accumulation wall. In a study on negatively charged polysaccharides it was found that when pure water was used as the carrier the retention levels were very low whereas they increased remarkably when salt was added [37]. The low retention was explained as an exclusion from the accumulation wall probably of electrostatic origin. This observation is however the opposite to that for glutenin in this study. A reason for the behaviour observed in the present study could therefore be the presence of a complex formation between the SDS and the protein. In the absence of SDS in the carrier the SDS–protein complex, formed in the dissolution procedure, may start to dissociate with a decreased solubility of glutenin as a result and perhaps followed by attractive interaction with the accumulation wall.

Previous results have suggested that the onset of overloading effects in FFF occurs at lower sample concentrations when the ionic strength is very low and that the effects could be reduced somewhat by increasing the background electrolyte concentration [19,34]. In reported cases of particle–wall interactions [17,34,38–41] an ionic strength of the carrier $\geq 10^{-3}$ M has been demonstrated to minimise these interactions. The 0.1% SDS used in the analysis of glutenin was therefore considered to be high enough to minimise overloading in the FFF channel. The experimental difficulties, in terms of foaming and disturbed detector signals, experienced at 0.1% SDS, further contributed to the rejection of even higher SDS concentrations. The complete absence of SDS in the carrier was however found not to be suitable either in the analysis of the glutenin proteins. The presence of SDS was necessary both to modulate the ionic strength and probably also to keep the sample as an SDS–protein complex. Any analyses of the sonicated samples in the absence of SDS were therefore not performed.

5. Conclusions

Overloading is a general problem when analysing ultra large macromolecules with FFF. In this study it was shown that the ultra large protein glutenin is best analysed using as low sample concentration as possible, rather injected in a large sample volume of low concentration than in a small volume of high concentration. It is also recommended to perform the fractionation at a low retention level, using only a moderate compression of the sample zone. The study also confirmed the need for a direct size determination method, such as light scattering, in combination with the FFF separation method, in order to accurately determine the size of ultra large macromolecules such as glutenin. The use of the retention time for any accurate size estimations, as in some previous studies on glutenin [6,7,22], requires that a linear FFF operation can be secured. As shown by this study it is a demand not easily fulfilled.

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