

Factors Influencing the Characterization of Gluten Proteins by Size-Exclusion Chromatography and Multiangle Laser Light Scattering (SEC-MALLS)¹

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ABSTRACT

Cereal Chem. 78(5):608–618

The use of multiangle laser light scattering (MALLS) in conjunction with size exclusion chromatography (SEC) was investigated for characterizing wheat proteins. Four solvent systems including 50% acetonitrile and 0.1% trifluoroacetic acid, 50 mM sodium phosphate (NaPhos) pH 2.5, 500 mM acetic acid, and 50 mM NaPhos pH 7.0 + 1% SDS were evaluated for protein extraction as well as for use as SEC mobile phases for MALLS analysis. The dn/dc values for wheat proteins were measured in each solvent. Except for the SDS-based mobile phase, gluten proteins showed dn/dc values of 0.16–0.20 that were similar to values reported for other proteins. When analyzed in the SDS solvent, gluten proteins showed

dn/dc values of 0.32, similar to that found for other SDS-protein complexes. While all solvents showed similar resolution when used as mobile phases in SEC analysis, the SDS solvent extracted the most protein (≈82%) in the unreduced form. This solvent system also displayed no concentration-dependent or electrostatic effects during MALLS analysis. SDS-soluble and insoluble protein complexes were characterized by MALLS. M_w distributions of 8.1×10^7 Da were found for the SDS-insoluble protein complexes. The effect of the column void volume was also examined as was data analysis parameters such as fitting method and peak placement.

The unique properties of gluten proteins are primarily responsible for the ability of wheat flour to be formed into viscoelastic doughs. Gluten proteins of wheat can be divided into two major groups. Gliadins are monomeric proteins and glutenins are large polymeric proteins formed by intermolecular disulfide bonds.

The large polymeric glutenins have been given several different names that are often dependent on the sample handling preparation protocol being followed (e.g., acetic acid insoluble proteins). These protein complexes are perhaps the largest found in nature (Wrigley 1996) and have often been correlated with breadmaking properties such as mixing time, extensibility, and loaf volume (Orth and Bushuk 1972; Chakraborty and Khan 1988; Dachkevitch and Autran 1989; Singh et al 1990; Gupta et al 1993; Bean et al 1998; Sapirstein and Fu 1998) and durum pasta quality (Sgrulletta and De Stefanis 1989).

Because of the correlation to functionality, much research has been focused on the structure, composition, and size of these polymeric proteins. It is often stated that the molecular weight distribution of the glutenin polymers is related to their functionality (Wrigley and Bietz 1988; MacRitchie 1992; Southan and MacRitchie 1999). However, no direct method for measuring true molecular weights of the polymers is available.

Recently, multiangle laser light scattering (MALLS) was used to characterize glutenin proteins in conjunction with reversed-phase (RP) HPLC (Lookhart 1997). The combination of HPLC and MALLS is a quick, reliable technique for determining the molar mass and radius of gyration of macromolecules (Wyatt 1993; Wen et al 1996; Folta-Stogniew and Williams 1999). Both RP-HPLC and size-exclusion chromatography have successfully been used with MALLS to characterize a wide range of proteins (Astafieva et al 1996; Jumel et al 1996; Wen et al 1996; Zhu et al 1996; Folta-Stogniew and Williams

1999; van Dijk and Smit 2000). When properly used, MALLS is capable of accurately determining the M_w of proteins. Zhu et al (1996) and Folta-Stogniew and Williams (1999) reported the M_w measurement of 22 proteins by MALLS with average error ranges from predicted M_w values of 0.3–3.2%.

In SEC-MALLS, proteins are first separated by size (hydrodynamic radius, not simply mass) (Potschka 1988) and then passed through the MALLS detector where laser light scattered by the macromolecules is recorded by photodiodes placed at multiple angles around a flow cell (Wyatt 1993). The concentrations of each eluted component (or time slice) are measured by a differential refractive index (DRI) detector located downstream from the MALLS detector.

The scattered light, or excess Rayleigh ratio (R_0), is related to the weight-average molecular mass of the macromolecules:

$$\frac{R(\theta)}{K^*c} = M_w P(\theta) [1 - 2A_2cM_w P(\theta)] \quad (1)$$

where c = concentration, M_w = molecular weight average molar mass, $P(\theta)$ = the scattering form factor, and A_2 = second virial coefficient (Wyatt 1993). This equation is valid only for molecules in suspension and whose refractive index is similar to that of the solvent of the suspended molecules (Wyatt 1992). Under typical chromatographic conditions, the term A_2c is often assumed to be zero due to the low concentrations of the analytes present (Wyatt 1993). Specifically, this assumption holds true when the term A_2c is very small relative to $1/M_w$ (Wyatt 1993). Other researchers have reported that protein concentration should be kept <2.5% (w/w) protein (van Dijk and Smit 2000) or <3% of the column volume filled with sample (Folta-Stogniew and Williams 1999).

K^* is the optical constant defined by:

$$K^* = \frac{4\pi^2 n_0^2}{\lambda^4 N_A} \left(\frac{dn}{dc} \right)^2 \quad (2)$$

with n_0 = refractive index of the solvent, N_A = Avogadro's number, λ = the wavelength in a vacuum of the light used, and dn/dc = the specific refractive index of the analyte (Zhu et al 1996). For indepth discussions on the theory of HPLC-MALLS, readers are directed to other excellent discussions (Wyatt 1993; Astafieva et al 1996; van Dijk and Smit 2000).

Note that the measurements produced by MALLS are absolute measurements; no calibration curves are generated from standard proteins (Wyatt 1993). Thus MALLS should prove to be extremely useful in the characterization of cereal polymeric proteins and how they relate to functionality in foods. However, extreme care should be taken when using MALLS to ensure that all procedures

¹ Cooperative investigations, U.S. Department of Agriculture, Agricultural Research Service, and the Department of Grain Science and Industry, Kansas State University, Contribution 01-243-J, Kansas State Agricultural Experiment Station, Manhattan, KS 66506.

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³ USDA-ARS, Grain Marketing and Production Research Center and Kansas State University. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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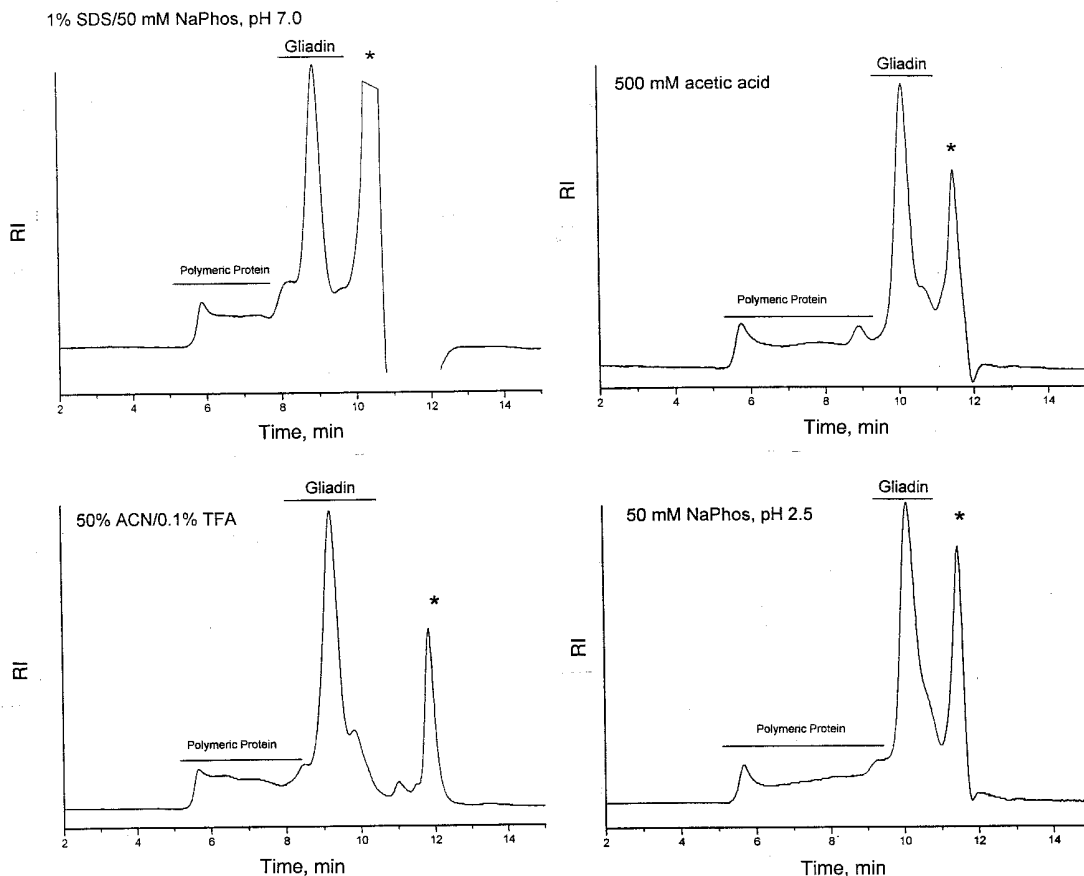


Fig. 1. Effect of mobile phase on resolution of size-exclusion chromatography (SEC) separations. * = solvent peak truncated in SDS mobile phase to allow for better comparison of protein peaks to over separations. All mobile phases were used with a Biosep SEC4000 column at a flow rate of 1.0 mL/min and at 40°C.

TABLE I
Amount of Protein (% of total) Extracted Using Various Solvents and Extraction Times

Solvent ^a	Short-Term ^b	Long-Term ^c	Sequential ^d
500 mM acetic acid	58.8 ± 0.01	74.8 ± 0.02	74.6 ± 0.01
1% SDS/NaPhos, pH 7.0	66.3 ± 0.02	80.0 ± 0.02	81.4 ± 0.04
50 mM NaPhos, pH 2.5	56.3 ± 0.03	66.3 ± 0.02	73.7 ± 0.01
50% ACN/0.1% TFA	57.4 ± 0.01	60.1 ± 0.02	64.2 ± 0.03

^a NaPhos = sodium phosphate, ACN = acetonitrile, TFA = trifluoroacetic acid.

^b Two 5-min extractions pooled 1:1.

^c Single 24-hr extraction.

^d Two 5-min extraction followed by a single 24-hr extraction.

are well controlled and that several critical factors are correctly addressed. This article reports on the assessment of SEC-MALLS for the characterization of wheat gluten proteins and investigates critical factors such as dn/dc, mobile phase composition, and sample extraction and preparation.

MATERIALS AND METHODS

SEC-MALLS

A Hewlett-Packard 1090 HPLC instrument was used and all samples were separated with a Biosep SEC-4000 column (Phenomenex, Torrance, CA). In some cases, a Waters ProteinPak 300SW column (Waters Corp. Milford, MA) was used for comparison. Column temperature was maintained at 40°C. Several mobile phases were used including; 50 mM Naphos, pH 7.0, + 1% SDS, 500 mM acetic acid, 50 mM Naphos, pH 2.5, and 50% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA). Mobile phases were vacuum filtered and degassed using a Solvac filtration system (Gelman, Ann

TABLE II
dn/dc Values^a for Different Solvent and Mobile Phase Combinations

Solvent and Mobile Phase ^b	dn/dc	Standard Deviation (n = 2)
500 mM acetic acid	0.18	0.01
1% SDS/NaPhos, pH 7.0	0.31	0.01
50 mM NaPhos, pH 2.5	0.16	0.01
50% ACN/0.1% TFA	0.20	0.02

^a Specific refractive index of the analyte.

^b Solvent was used to both extract proteins and as mobile during SEC analysis. NaPhos = sodium phosphate, ACN = acetonitrile, TFA = trifluoroacetic acid.

Arbor, MI) with 0.2-μm filters (Millipore, Bedford, MA). Mobile phases were continuously sparged with helium during use. SDS mobile phases were vacuum filtered before the addition of SDS and were not helium sparged during use due to foaming of SDS. A guard column (Securityguard, Phenomenex, Torrance, CA) was used during all separations and an inline 0.5-μm filter (PJ Cobert, St. Louis, MO) was placed upstream of the guard column. A 0.1-μm filter (Millipore Corp. Bedford, MA) was also placed inline immediately after the high pressure pump.

MALLS data were gathered with a multiangle light scattering detector (DAWN EOS, Wyatt Technology Corp. Santa Barbara, CA) with 18 detection angles and a DRI (Optilab DSP). The voltages from the photodiodes at each scattering angle were normalized using bovine serum albumin (BSA). The light scattering detector was calibrated with toluene as recommended by the manufacturer. The delay volume between the light scattering detector and DRI was measured using BSA as a marker. The DRI detector voltage response was calibrated twice with five concentrations of sodium chloride and the temperature of the DRI detector was maintained at 40°C.

Determining dn/dc

Values for dn/dc were measured online as described in Astafieva et al (1996). Samples for the dn/dc measurements were prepared by extracting soluble proteins from six hard red winter wheats cultivars. All samples were extracted and analyzed in duplicate on three separate occasions. The protein extracted was quantified by determining the amount of insoluble protein as described in Bean et al

(1998) and subtracting from the total flour protein (extracted protein = total protein – insoluble protein) (Bean and Lookhart 1998). The dn/dc measurements were made on the entire SEC chromatogram and also separately on the polymeric protein and monomeric protein regions of the chromatograms. To quantitate the amount of polymeric and monomeric proteins, the percent of area of each region was determined from the UV data. This percentage was then multiplied by the amount of protein extracted (Bean et al 1998). The dn/dc values were also estimated from the amino acid compositions as described in Zhu et al (1996).

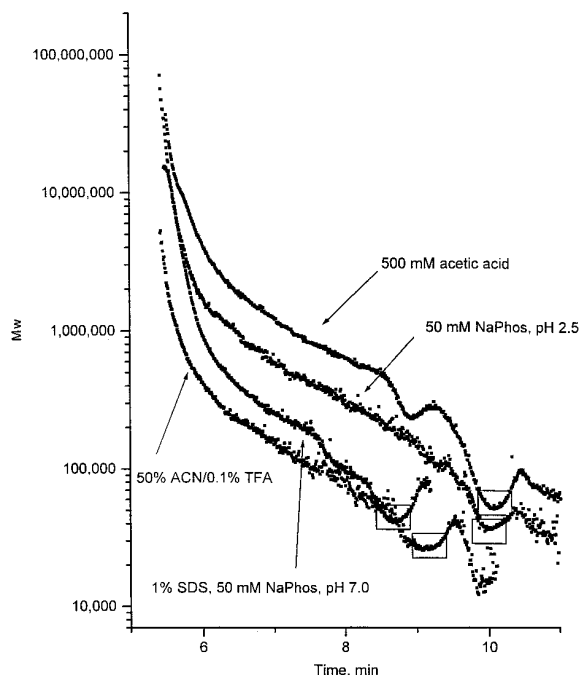


Fig. 2. Comparison of soluble proteins extracted and analyzed in each solvent. Samples were extracted with each solvent (short-term) and analyzed using that solvent as mobile phase. Separation flow rate 1.0 mL/min at 40°C. Box = approximate location of gliadin region in each separation.

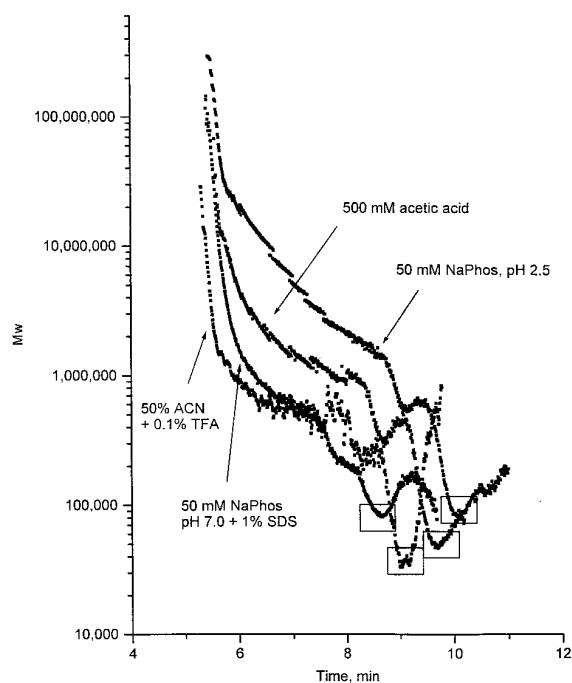


Fig. 3. Effect of solvent on lyophilized proteins resolubilized in each solvent and analyzed using that solvent as mobile phase. Separation flow rate 1.0 mL/min at 40°C. Box = approximate location of gliadin region in each separation.

Protein Extraction and Quantitation

Flour samples were extracted with various solvents including 50 mM Naphos, pH 7.0, + 1% SDS, 50% ACN and 0.1% TFA, 500 mM acetic acid and 50 mM Naphos, pH 2.5. Different solvent-to-flour ratios tested included 4:1, 10:1, and 20:1. All extractions were done using a vortex genie2 mixer (Fisher, Pittsburgh, PA) equipped with a 30-place foam vial holder. Short-term extractions were made using two 5-min extractions with the supernatant from each extract pooled in a 1:1 ratio. Extracts were vortexed continually during extraction (Bean et al 1998). Long-term extractions were made for 24 hr using repeated 5-min cycles of continual vortexing and rest. This was accomplished by connecting the mixer to a timer programmed for repeated 5-min on-off cycles. A sequential extraction procedure extracted samples twice with solvent using two 5-min extracts followed by a 24-hr extraction. The amount of protein extracted was quantified by dn/dc determinations. Soluble samples were filtered before analysis using 0.45- μ m syringe filters (Gelman Acrodisc, Ann Arbor, MI). Insoluble samples were extremely difficult to filter due to the high viscosity of these samples. These samples were clarified by centrifugation before analysis.

RESULTS AND DISCUSSION

Protein Extraction

To take advantage of the ability of MALLS to characterize the M_w of gluten proteins, a maximum amount of protein must be extracted in the unreduced state. Many different solvents have been used to extract gluten proteins in their unreduced state with varying degrees of effectiveness (Meredith and Wren 1966; Danno 1981; Wrigley and Bietz 1988; Weegels et al 1994). For MALLS, the proteins must be extracted in a solvent that will not interfere with subsequent analysis, preferably in a solvent that can also be used as mobile phase in SEC to ensure protein solubility during separation. For these considerations, solvents tested for use in extracting proteins and as mobile phase in SEC-MALLS analysis were 50% ACN + 0.1% TFA, 50 mM NaPhos, pH 7.0, + 1% SDS, 50 mM NaPhos, pH 2.5, and 500 mM acetic acid. Each of these solvents, or a variant thereof, has been used to extract or analyze gluten proteins by numerous researchers.

For each solvent, three extraction schemes were evaluated. A short-term extraction consisting of two 5-min extractions (pooled 1:1), a long-term single 24-hr extraction, and a sequential extraction scheme composed of consecutive short-term and long-term extractions.

Initially, all solvents were tested at a flour-to-solvent ratio of 4:1. At this ratio, all solvents showed approximately the same amount of protein extracted. Increasing the solvent-to-flour ratio from 10:1 to 20:1 increased the amount of protein extracted but little difference was seen between these two ratios (data not shown). Thus, a ratio of 10:1 was used for the remainder of the study.

For the short-term extractions, the SDS solvent extracted the most protein, removing \approx 66% (Table I). The other solvents extracted between \approx 55–60%. The 24-hr long-term extraction removed more protein than the short-term extraction with the SDS solvent extracting \approx 80% while the other solvents extracted 60–75%. When these extractions were combined into a sequential extraction procedure, slightly more protein was extracted with the SDS solvent at \approx 82%.

Although the sequential extraction procedure showed only slight increases in the amount of protein extracted, this procedure was adopted for the remainder of the study. This procedure allowed the polymeric proteins to be divided into two classes: the easily extracted (soluble) and the difficult to solubilize (insoluble).

Several researchers (Danno 1981; He et al 1991) have reported protein extraction levels of $\approx 100\%$. However, with the sample used in these tests, we were unable to achieve this level of protein extraction. Excessive shear force was not applied during extraction because such forces may break some covalent bonds (MacRitchie 1975; Singh et al 1990; Weegels et al 1994) and thus alter the molecular weight distribution of the polymeric proteins. Such forces could be caused by sonication or through the use of a Waring blender, which is often used to extract proteins from flour or gluten (Meredith and Wren 1966; Danno 1981). Meredith and Wren (1966) reported no change in M_w with the use of a Waring blender. However, this needs to be investigated with the use of MALLS.

Mixed solvents (50% 1-propanol + SDS) were tested at SDS levels of 1–5%. No increase in extracted protein over that of 50% 1-propanol was observed (data not shown). This may have been caused by the organic solvents disrupting the SDS binding to the proteins (Bean et al 2000) and preventing the SDS from functioning efficiently.

dn/dc

Although SDS clearly was the most effective solvent tested for extracting cereal proteins, we also wished to test the effect of each solvent on the molecular weight distributions of the extracted proteins. However, to obtain this information from MALLS, the dn/dc of the proteins under the exact conditions (in each mobile phase) of analysis must be obtained (Huglin 1972; Eisenberg 1976). The dn/dc, or the change in refraction with the change in concentration, is used in Equation 2 and, therefore, directly influences the M_w determined from light scattering experiments.

To measure dn/dc of wheat proteins, six HRW wheats were analyzed by SEC-HPLC. The dn/dc was estimated as described in Astafieva et al (1996). Each sample was extracted in duplicate, and nitrogen combustion was used to accurately determine the amount of protein extracted. This procedure was repeated three times. The dn/dc values for each solvent are shown in Table II. Note that this represents samples extracted in these solvents and then analyzed using the same solvent as mobile phase in the SEC analysis. For 500 mM acetic acid, 50% ACN-TFA, and 50 mM NaPhos, pH 2.5, dn/dc values were 0.18–0.20.

Note that dn/dc was measured on the entire SEC chromatogram and separately for the polymeric proteins and gliadin regions. Polymeric proteins and gliadins were defined based on previous work (Dachkevitch and Autran 1989; Batey et al 1991; Larroque et al 1997). Peak regions for each solvent are shown in Fig. 1. In all cases, the values for polymeric proteins (mainly glutenins) and the gliadins were essentially identical (data not shown). This is not surprising, given the similarity in the amino acid composition between these protein fractions (Lookhart and Bean 2000) and the fact that aggregative status does not influence dn/dc (Eisenberg 1976).

The values shown in Table II for 500 mM acetic acid, 50% ACN-TFA, and 50 mM NaPhos, pH 2.5, corresponded well with dn/dc values of 0.16–0.19 reported for other proteins (Takagi 1981; Mhatre et al 1990; Mhatre and Krull 1993; Astafieva et al 1996; Jumel 1996; Zhu et al 1996; Folta-Stogniew and Williams 1999; van Dijk and Smit 2000).

The dn/dc values reported here are slightly higher than those reported earlier for gliadins (Robertson and Greaves 1911), where values of 0.15–0.17 were obtained in a number of typical gliadin solvents. An abnormally low value (0.04) was obtained by these authors in a solvent of 75% phenol. Phenol, however, has a much different refractive index (Robertson and Greaves 1911) than the other solvents tested and is therefore not directly comparable. Given the fact the sample preparation methods used by Robertson and

Greaves (1911) varied considerably from those of this study, and that dn/dc is sensitive to the solvents, the wavelength of the refractive index detector used, and the temperature of the measurements, the results in Table II agree fairly well with those measured in 1911.

The dn/dc values measured in the SDS solvent were much higher than in the other solvents (Table II). However, SDS is known to bind to proteins (Reynolds and Tanford 1970; Bietz and Wall 1972; Samsó et al 1995). Thus, when analyzing proteins exposed to SDS,

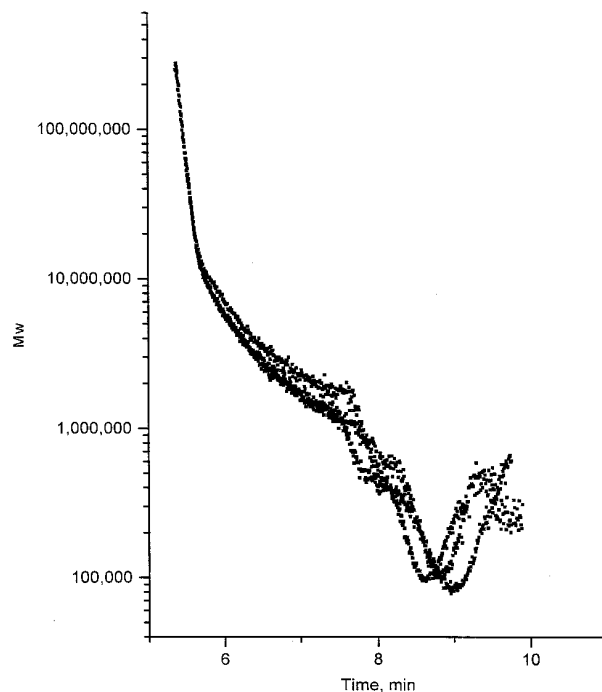


Fig. 4. Effect of NaCl on M_w distribution in samples extracted and analyzed using 50 mM NaPhos, pH 7.0, + 1% SDS in 0–80 mM NaCl. Separation flow rate 1.0 mL/min at 40°C.

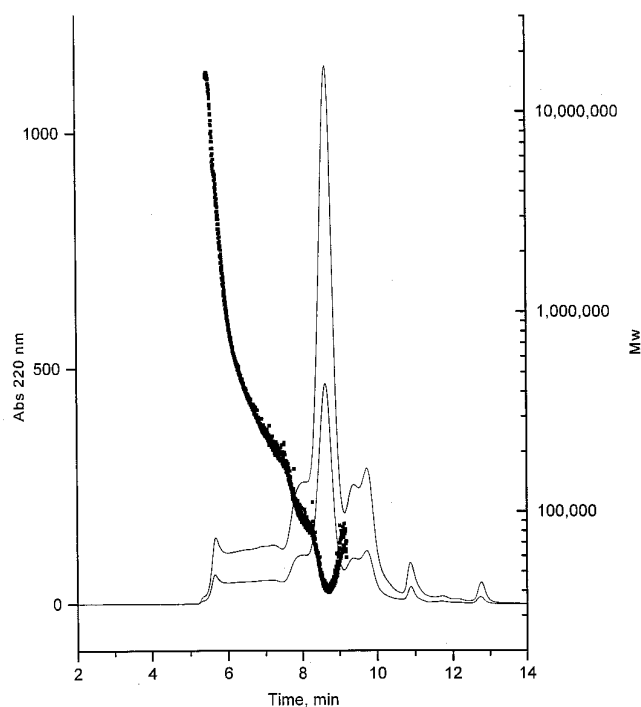


Fig. 5. Effect of concentration of samples extracted and analyzed using 50 mM NaPhos, pH 7.0, + 1% SDS at injection levels of 25 and 50 μ L. Separation flow rate 1.0 mL/min at 40°C.

the dn/dc of the protein-SDS complex, not of the protein alone, must be considered (Kameyama et al 1982). It should be stressed that subsequent measurements in the SDS mobile phase would reflect the M_w measurements of SDS-protein complexes, not of the proteins alone.

The values in Table II for the SDS-gluten protein mixture were similar to those reported for other proteins analyzed in SDS (Takagi 1980; Miyake and Takagi 1981; Kameyama et al 1982). These authors reported dn/dc values of 0.28–0.45 for several proteins in various levels of SDS. The dn/dc values were related to the amount of SDS bound to the proteins (Miyake and Takagi 1981; Kameyama et al 1982), which accounted for the rather large range of dn/dc values. For the six wheat cultivars examined here, however, the standard deviation for dn/dc was only $\approx 3\%$, which suggests that there was little variation in the amount of SDS bound between cultivars.

The dn/dc of proteins extracted with SDS but then analyzed in a non-SDS mobile phase (50% ACN-TFA) showed dn/dc values of 0.31. This was probably due to residual SDS bound to the proteins. Thus, it is extremely important to measure dn/dc under the exact conditions that will be used for analysis. It is also important to consider the effect of any compound such as detergent or salt that might bind to the proteins either during analysis or extraction.

Estimates of the dn/dc values of proteins can be made based on amino acid composition (Zhu et al 1996). Estimates of dn/dc for total gluten, gliadin, and glutenin all showed values of ≈ 0.22 g/mol. These estimations are slightly higher than the dn/dc values measured online. The estimation uses information from the partial molar volumes of the amino acids, in water, calculated at the wavelength of sodium light (Zhu et al 1996). Because these factors were different from the actual experimental conditions used to analyze wheat proteins, the estimates may vary slightly from actual. Thus, overall, these estimations agree with the measured values. Also, note that the estimates were the same for gluten, gliadin, and glutenin, which agreed with the data from the online measurements.

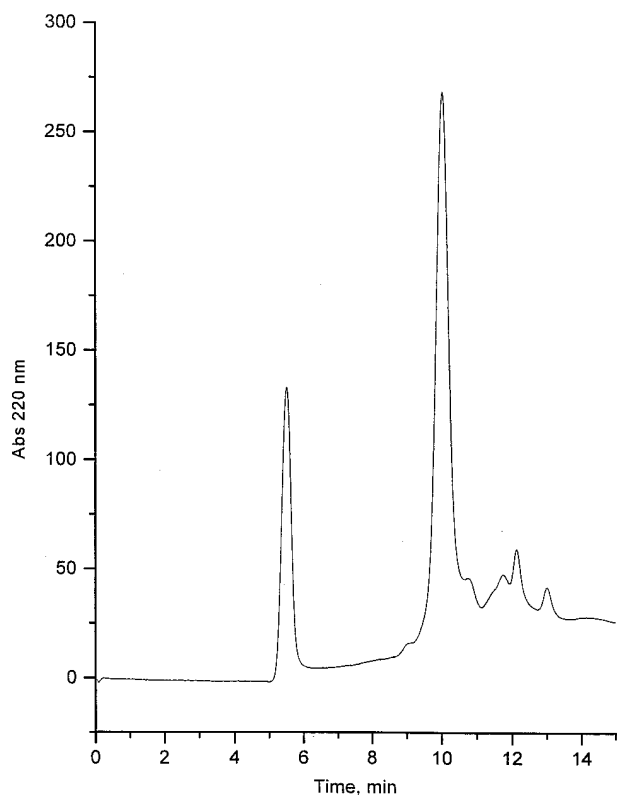


Fig. 6. Effect of injecting a sample extracted using 50 mM NaPhos, pH 7.0, + 1% SDS into a mobile phase consisting of 50 mM NaPhos, pH 2.5 Separation flow rate 1.0 mL/min at 40°C.

Analysis Conditions and Effects on M_w Measurements

Once dn/dc values were established for each solvent system, it was possible to make comparisons of the molecular weight distributions of the proteins extracted by each solvent. First, samples were extracted with each solvent and analyzed using the extraction solvent as mobile phase. The resolution between each mobile phase was roughly equal (Fig. 1), allowing comparison of the separations in each mobile phase.

Because the resolution was similar in each mobile phase, it was possible to compare the M_w distributions in each solvent and mobile phase system (Fig. 2). Note that the elution times shift slightly between mobile phases, which complicates the direct comparisons of the M_w distribution curves in Fig. 2. This is most evident in the gliadin regions, where elution times range from ≈ 9 to 10.5 min (Fig. 1). While the gliadin regions differed slightly from mobile phase to mobile phase, it was easy to identify the major gliadin peak.

Even with slight differences in elution times, it was readily apparent that the polymeric proteins displayed wide variations in M_w distributions. There could be several reasons for this. Each solvent

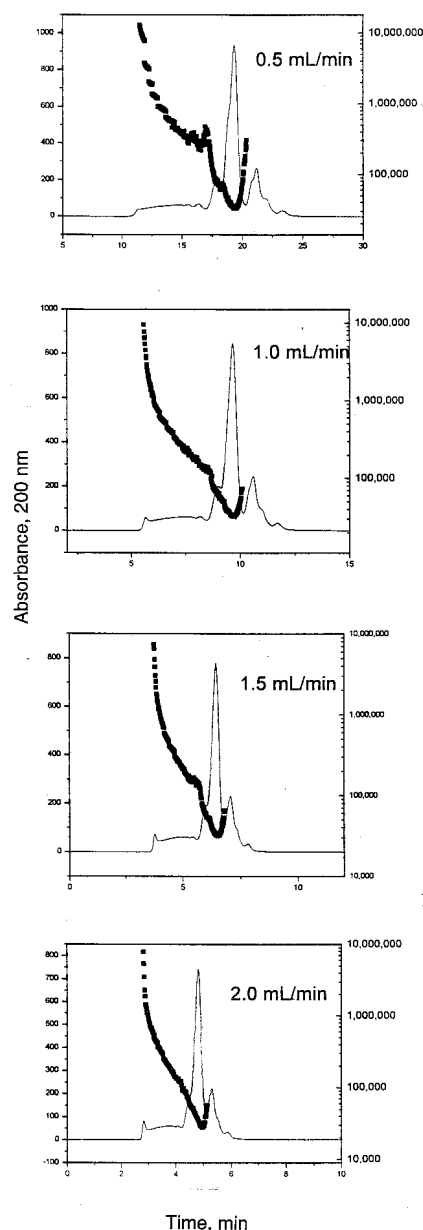


Fig. 7. Effect of flow rate on M_w distribution of wheat proteins separated on Biosep SEC4000 column at flow rates of 0.5, 1.0, 1.5, and 2.0 mL/min at 40°C.

could have extracted a different range of polymeric proteins. Different solvents may aggregate or disaggregate the proteins through noncovalent interactions to different degrees, thus influencing the M_w distributions. Each solvent could also affect the hydrodynamic volume and thus the resolution of the polymeric proteins, such that different hydrodynamic volumes or M_w ranges were being separated at any given point, thus producing different average M_w distribution curves for each solvent.

To examine the effect of the solvent, we compared the measured M_w for the gliadin regions of each separation, as this region should contain the same set of proteins. The resulting average M_w were 49.2 kDa (SDS), 30.2 kDa (ACN), 41.7 kDa (NaPhos, pH 2.5), and 68.1 kDa (acetic acid). From these data, it was clear that the separation solvent has an effect on the measured M_w distributions.

Because each solvent could selectively extract different groups of polymeric proteins and have different effects on the aggregation of the polymeric proteins, further attempts were made to examine the effects of the solvent on M_w distribution. Samples were extracted with 50% 1-propanol and lyophilized. The lyophilized samples were then redissolved in each solvent and analyzed. This experiment allowed the same set of proteins to be compared in each solvent system. As a control, samples were also redissolved in 50% 1-propanol and compared with the original extract. The molecular weight distributions of the original and lyophilized samples solubilized in 50% 1-propanol were essentially identical, showing that the lyophilizing process did not adversely affect the proteins (data not shown).

When the M_w distributions from the lyophilized samples resolubilized by the various solvents were compared, the polymeric proteins in each solvent system again displayed different M_w distributions (Fig. 3). However, in this analysis, the proteins redissolved and analyzed in 50 mM NaPhos, pH 2.5, contained polymeric proteins with the highest M_w distributions. The differences in the M_w distributions between Figs. 2 and 3 were probably due to the size distribution and aggregation states of the polymeric proteins extracted with each solvent. Each solvent clearly extracted different amounts

of proteins (Table I) and therefore would be expected to extract a different size range of polymeric proteins. This difference is reflected in Fig. 2. However, each solvent may also have an effect on the aggregation state of the polymeric proteins, which is reflected in Fig. 3, where the same set of proteins were redissolved in each solvent.

The solvent used in MALLS analysis can influence the M_w distributions in several ways. First, different solvents may influence the aggregative state of the polymeric proteins to different degrees. The SDS solvent may be best at disrupting noncovalent interactions and reducing hydrodynamic volume, which may be one reason why the lowest M_w polymers were found in the SDS-based mobile phases. The SDS solvent is at pH 7.0, while the other solvents are at acidic pH levels. This in turn could influence the aggregative state of the proteins.

The mobile phase may also have influenced the light scattering data directly. Strictly speaking, light scattering is only valid for two-component systems (Nagasawa and Takahasi 1972; Straizelle 1972; Eisenberg 1976; Mhatre et al 1990; Astafieva et al 1996). This is not typically the case in protein separations with several components such as protein, buffer salts, solvents, and detergents. (Burchard and Cowie 1972; Strazielle 1972; Eisenberg 1976; Mhatre et al 1990). However, light scattering in multicomponent systems can be accomplished if the components are iso-refractive (Mhatre et al 1990), which is the case with acetonitrile-water mixtures (Astafieva et al 1996). However, measuring the dn/dc of the proteins under the exact analysis conditions can minimize the effect of the additional components and interference of small molecules and the need for additional terms in the light scattering equation (Eisenberg 1976).

The pH level of the mobile phase is also important because electrostatic effects can also influence the M_w measured by light scattering. For this reason, it is often recommended that proteins be analyzed as close to neutral as possible (Burchard and Cowie 1972; Nagasawa and Takahasi 1972; Straizelle 1972; Eisenberg 1976). For

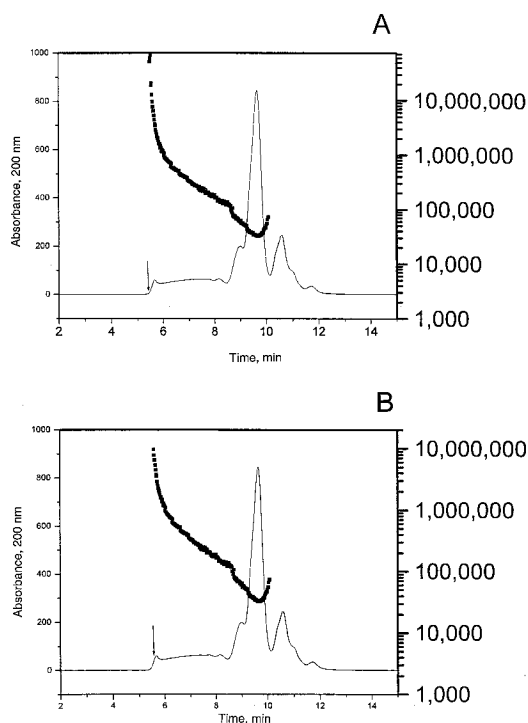


Fig. 8. Effect of peak placement on M_w distribution curves. **A**, Peak started at beginning baseline of differential refractive index (DRI) detector sample. **B**, Peak started with signal-to-noise >2 (exaggerated to visualize effect on M_w distribution curve). Separation flow rate 1.0 mL/min at 40°C.

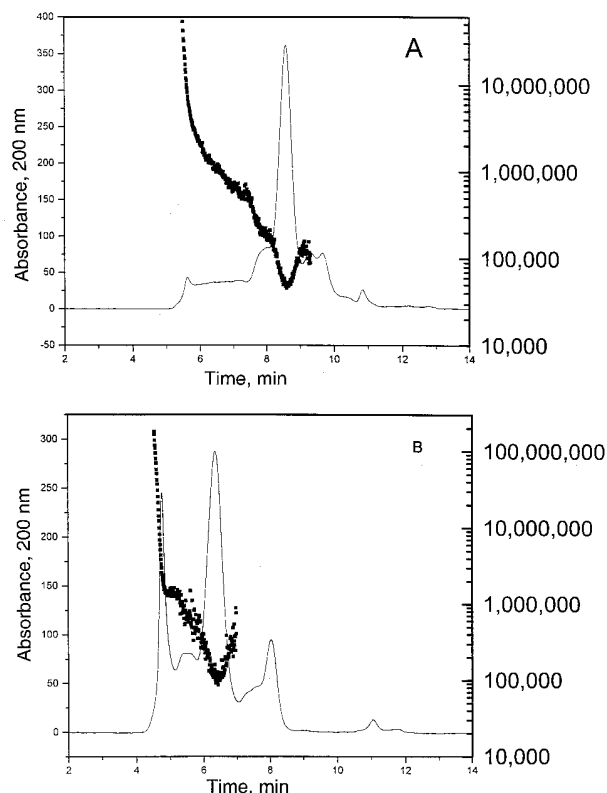


Fig. 9. Effect of column exclusion limit on M_w distribution curve. Separation flow rate 1.0 mL/min at 40°C using **A**, Biosep SEC4000 column and **B**, Waters ProteinPak 300SW column.

wheat proteins, however, this would be virtually impossible. Many wheat proteins are not soluble (especially in unreduced form) except in a solvent that produces a net charge on the protein or in charged detergents or other compounds that can bind to the proteins (MacRitchie 1992). This means that complex (multicomponent) solvents are needed to solubilize and analyze wheat proteins. It is not possible to analyze wheat proteins in ideal conditions for light scattering analysis. Note that these considerations are important in any separation mechanism like SEC or other techniques such as field flow fractionation (FFF).

The impact of several of these factors can be minimized by choosing appropriate conditions for analysis. To minimize electrostatic effects, proteins should be analyzed in low levels of neutral salts (Burchard and Cowie 1972; Nagasawa and Takahasi 1972; Strazielle 1972; Eisenberg 1976). Therefore, the effect of added salt was tested using 50 mM NaPhos, pH 7.0, + 1% SDS mobile phase.

For the SDS mobile phase, no effect on the M_w distribution was seen when proteins were analyzed in three different levels of NaCl (0–80 mM) (Fig. 4). This may be due to the fact that this mobile phase already contains relatively high levels of Na^+ ions from the NaPhos buffer as well as from SDS. The elution times of the gliadin peaks changed slightly between the salt levels, which accounts for the slight variation in the M_w curves at 8–10 min. Similar results were seen when NaI was used in place of NaCl (data not shown).

While no effect was seen in the SDS mobile phase, Burchard and Cowie (1972) cautioned that in low ionic strength mobile phases, errors in M_w of up to 100% were possible; this included the use of organic acids such as acetic acid. Thus, when using a low ionic strength mobile phase, the effect of salts must be carefully studied. This factor could account for some of the differences in the M_w distributions seen in Figs. 2 and 3 between the various mobile phases and may be one reason why the M_w for gliadins were much higher in the acetic acid mobile phases than all the other solvents tested.

Another important consideration is the effect of the A_2c term in

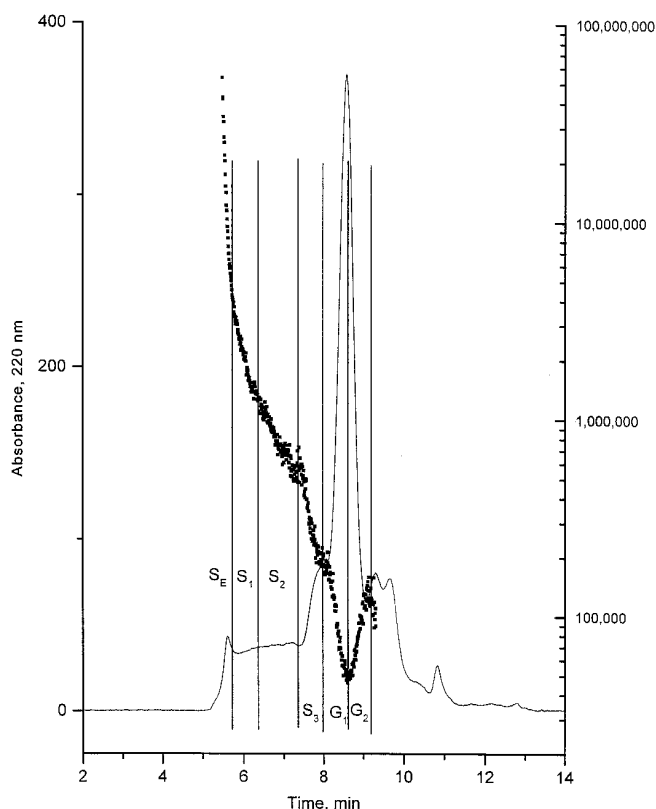


Fig. 10. Peak regions for soluble proteins. Separation flow rate 1.0 mL/min at 40°C.

Equation 1. This is often assumed to be zero for SEC separations due to the low levels of proteins actually injected into SEC columns during analysis (Wyatt 1993). However, several authors have suggested that protein levels be kept low during analysis to avoid this problem (Jumel et al 1996; Folta-Stogniew and Williams 1999; van Dijk and Smit 2000). To verify this assumption under the conditions used in this study, two different amounts of proteins were injected and the M_w distribution was measured (Fig. 5). No concentration-dependent effects were seen at these levels, indicating that the A_2c term can be considered zero.

Based on these factors, the use of 50 mM NaPhos, pH 7.0, + 1% SDS was selected for routine use and further study. This solvent extracted the most protein (Table I), showed no electrostatic effects on M_w (Fig. 4) and also showed no concentration dependent behavior (Fig. 5). In addition, this solvent has been widely used in the analysis and extraction of wheat proteins (Danno 1981; Bietz 1984; Dachkevitch and Autran 1989).

Finally, it is also important to consider possible interactions between sample preparation and separation methodology. For example, when wheat flour samples extracted (short-term) in 50 mM NaPhos, pH 7.0, + 1% SDS were injected into the 50 mM NaPhos, pH 2.5, mobile phase (Fig. 6), the M_w distribution of the soluble polymeric proteins showed M_w at $>10^8$ Da (data not shown). Comparing the chromatogram to previous separations in the SDS mobile phase (Fig. 2) showed virtually all of the polymeric protein was present at the void volume, suggesting that severe aggregation had occurred. This aggregation may have been due to the exposure of the protein hydrophobic cores in SDS which were then allowed to interact, once injected into the aqueous environments of the acidic mobile phase. Thus, it is important to carefully consider sample preparation and analysis conditions when preparing samples for MALLS analysis. The use of salt to precipitate gluten proteins as a preparative method (Wrigley and Bietz 1988) could possibly produce aggregation which could, in turn, lead to altered M_w distributions when analyzed by MALLS. However, this does bring up the possibility of using MALLS to study noncovalent interactions in wheat proteins.

It is also necessary to consider sample solubility and mobile phase composition. For example, when trying to analyze long-term SDS extracts in the 50% ACN and 0.1% TFA mobile phase, we found that column back pressures increased rapidly. This was probably due to insolubility of the large insoluble proteins in the ACN mobile phase. Batey et al (1991) reported that SDS extracts showed no precipitation when mixed with ACN mobile phases. However, in that experiment, the samples used would roughly correspond to the soluble proteins (short-term extracts). When these samples were analyzed in the ACN mobile phases, no pressure problems were noted. It was only when the larger proteins (long-term extracts) were analyzed that increased back pressure and guard column plugging

TABLE III
Comparison of Fitting Algorithms on M_w Measurements

Algorithm	SE	S1	S2	S3	G1	G2
Berry	1.58×10^7	3.53×10^6	1.42×10^6	4.08×10^5	1.25×10^5	1.22×10^5
Debye	1.07×10^7	2.35×10^6	9.32×10^5	3.03×10^5	1.07×10^5	1.08×10^5
Zimm	2.55×10^7	5.16×10^6	2.14×10^6	5.16×10^5	1.37×10^5	1.34×10^5

TABLE IV
Different Molecular Weight Averages for SDS Soluble Proteins^a

Mode ^b	SE	S1	S2	S3	G1	G2
M_n	6.87×10^6	3.30×10^6	1.30×10^6	3.77×10^5	1.18×10^5	1.11×10^5
M_w	1.58×10^7	3.53×10^6	1.42×10^6	4.08×10^5	1.25×10^5	1.23×10^5
M_z	4.61×10^7	3.77×10^6	1.57×10^6	4.52×10^5	1.54×10^5	1.37×10^5
M_w/M_n	2.29	1.07	1.10	1.08	1.16	1.10

^a All values calculated with the Berry fitting method.

^b M_n = molecular number average, M_w = molecular weight average; M_z = molecular Z average, M_w/M_n = polydispersity.

problems were found. This solubility problem is also suggested from the data in Table I. The SDS solvent clearly extracted much higher levels of protein than did the ACN in the long-term and sequential extractions. Because the material in these extracts is mainly polymeric protein, the solubility is clearly lower in the ACN solvent for these proteins. Sample solubility must be considered when analyzing wheat protein extracts by SEC-MALLS.

The final analysis condition studied was the effect of flow rate on the M_w distribution of wheat proteins. Throughout this study, a

flow rate of 1.0 mL/min was used in the SEC analysis. Recently it was demonstrated that flow rates with the type of column used in this study could be increased to 2.0 mL/min to produce more rapid separations (Larroque and Bekes 2000). However, large polymers can suffer from shear degradation during SEC analysis, and high flow rates may increase this problem (Barth and Carlin 1984). Thus, the effect of flow rate was examined on the M_w distribution of the soluble proteins. As flow rate increased, the resolution in the polymeric region slightly decreased and the M_w distribution curves became much steeper (Fig. 7). However, no large changes in the M_w ranges were seen, indicating that shear degradation was not a problem at the higher flow rates. The same results were seen with the insoluble proteins (data not shown). Based on the balance between resolution and separation time, 1.0 mL/min was chosen for routine use.

Effect of Peak Placement and Void Volume on M_w Distributions

While MALLS produces absolute M_w measurements without the need to calibrate with standard proteins, several data analysis factors must be correctly addressed. One important factor is the placement of the peaks during analysis. Both the MALLS and the DRI signals must be large enough for signal-to-noise ratios of at least 2 (Wyatt Technology Corp. 1997). This is particularly important for the DRI signal. The concentration must be known to accurately determine the M_w of a peak (Wyatt 1993). The DRI signal is used to determine the concentration, thus the placement of peak stop and start times must be selected so that adequate DRI signal is present.

To illustrate the effect of this on the M_w distribution of wheat proteins, two M_w distribution curves were generated with different peak start placements (Fig. 8). The first placement was selected to start at the very beginning of the DRI peak. The second was placed at an exaggerated position with a signal-to-noise ratio of $>2:1$. It can clearly be seen that the placement of the peak start position influences the upper range of the M_w distribution curve. Setting the peak start position where the DRI signal is too low to be accurate leads to inaccurate M_w distribution upper limits.

In SEC analysis, caution must be used in interpreting the M_w distributions in the exclusion limits of the columns. The maximum possible exclusion limit for modern SEC columns is ≈ 10 million

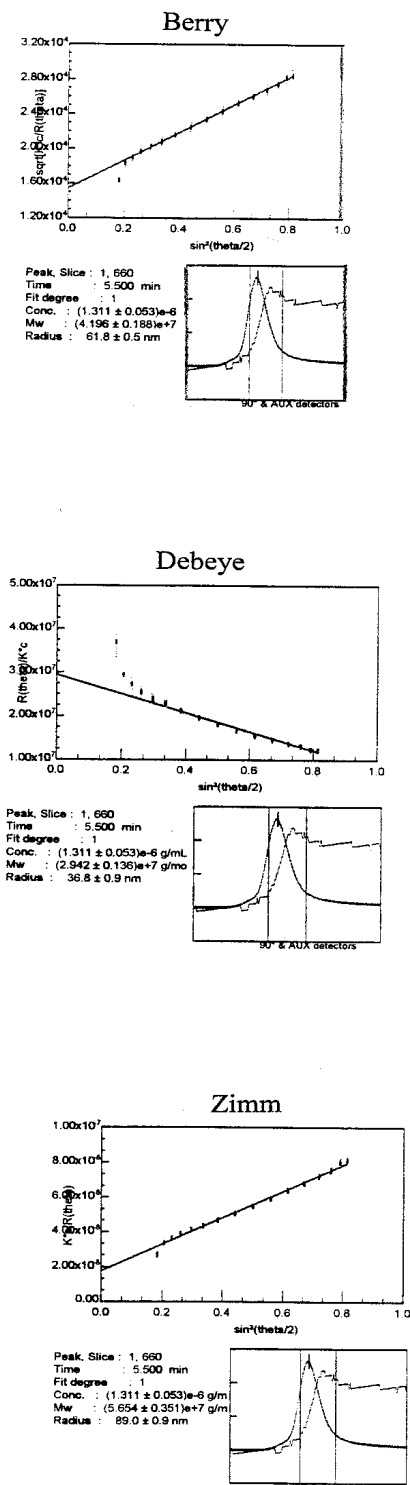


Fig. 11. Comparison of Debye plots for the Berry, Debye, and Zimm fitting methods.

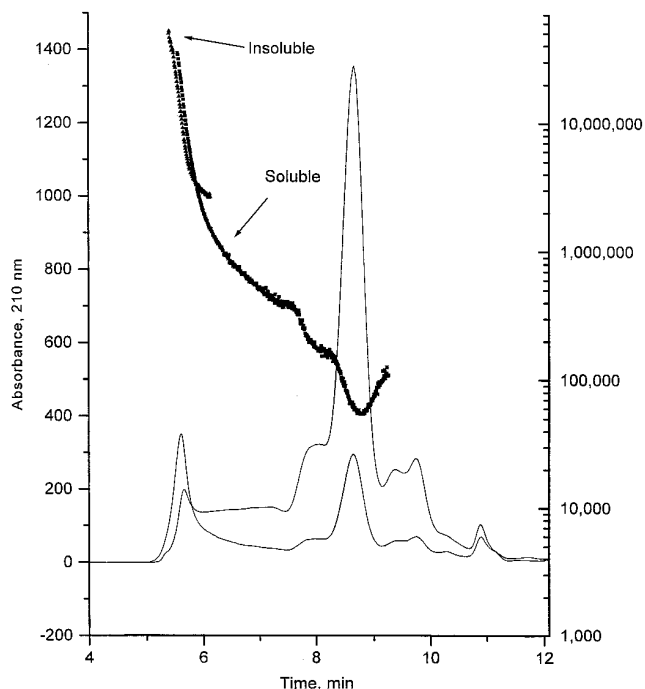


Fig. 12. Comparison of M_w distributions of SDS soluble and insoluble proteins.

Da (Jumel et al 1996) and material larger than this will be excluded. Excluded material is not separated, thus the M_w distribution curves in this region cannot be used (Jumel et al 1996). This means that the raw upper limit of the M_w curves should not be reported as the largest material extracted. The weight average M_w in the exclusion region is still valid, however (Jumel et al 1996).

The effect of this can be seen in Fig. 9 where the same extract was analyzed on two different columns with different nominal exclusion limits. The extract separated using a BioSep SEC-4000 (Fig. 9A) had an exclusion limit of 2,000 kDa for native proteins reported by the manufacturer. The extract separated using a Waters ProteinPak 300SW (Fig 9B) had a reported exclusion limit of 400 kDa for native globular proteins. If only the upper limits of the M_w distributions were considered, it would appear that the sample separated in the Waters column would contain larger proteins. As these were the same sample, this is clearly not the case. It should be emphasized that the exclusion limit will influence the proportion of protein excluded, which in turn will influence the average M_w of this material. Therefore, extreme caution should be used when comparing data analyzed on different types of columns.

Data Analysis and Interpretation

To analyze and interpret the M_w distribution from SEC separations, several peak regions were defined for both the soluble and insoluble fractions. An example of those regions selected for the soluble proteins is shown in Fig. 10. The first three regions were also used for the insoluble proteins, except they were labeled I_E , I_1 , and I_2 (data not shown). These regions were defined based on previous work (Dachkevitch and Autran 1989; Batey et al 1991; Larroque et al 1997) and on natural breaks in the M_w distribution curve. Note that at the end of the M_w curve in the gliadin region, the MALLS data indicates that the M_w increases sharply. The degree of this increase varied considerably from sample to sample (data not shown). The reason for this is not known. However, this type of curve has been reported before for samples of heparin. Wyatt (1992) reported that this upward swing could be due to branching, microgel components or local changes in dn/dc . Because albumins and globulins are found in this region of the SEC chromatograms, the upward swing could be related to changes in the dn/dc of these proteins relative to the gluten proteins. A sample of gliadins analyzed by SEC-MALLS without albumins and globulins did not show this increase in the M_w distribution curve (data not shown), which supports the idea that the albumin and globulin proteins cause this anomalous upward curve. This phenomenon using purified protein fractions should be researched further.

Once peak regions were defined, we could examine the effects of various data processing parameters on computed results. Several different fitting algorithms can be used to determine M_w distributions from light scattering data. The Berry, Zimm, and Debye methods were available with the software for the instrument used in this study. The Berry method has been useful for large molecules, while the Zimm method works very well for midsized molecules (root mean square radius 20–50 nm) (Jeng and Blake 1993). The Debye fitting method is useful over a wider range of molecules than the Zimm. The effect on the M_w for each peak region is shown in Table III. Considerable differences were noted for the M_w averages of the largest fractions S_E , S_1 and S_2 . The smaller M_w material, however, showed similar M_w averages.

Debye plots were also made for the S_E (excluded) region for each fitting algorithm (Fig. 11). With the Debye algorithm, more curvature could be seen at the low angles. This curvature could be due to the large M_w of this material or possibly due to a wide molecular weight distribution, particulate contamination, microgel formation, or a high degree of branching (Jumel et al 1996). There was little curvature with the Zimm and Berry fitting methods. While it was possible to use curve fitting to fit data from the Debye method, the more linear fits found with the Zimm or Berry methods may be preferable. For further work, the Berry method was chosen.

In addition to the weight average (M_w), number average (M_n), and the Z average (M_z) molecular weight distributions can be obtained from MALLS data. The values for the SDS soluble proteins are shown in Table IV. These averages emphasize different weight fractions (Southan and MacRitchie 1999). Except for the material eluting at the void volume (S_E), all values were very similar.

The polydispersity values were also calculated for each fraction (Table IV). All peak regions showed values of ≈ 1 except for the S_E fraction. This is the material eluted at the void of the column and, as mentioned previously, consists of unfractionated polymers and aggregates.

Protein Characterization

While the molecular weight distribution of the polymeric proteins of wheat has generally been considered a major quality factor, no direct method for measuring this distribution has been available. The use of selective solubility has been used in a number of studies to indirectly study the molecular weight distribution, with good results. This method assumes that the more readily soluble proteins are smaller in M_w than the insoluble proteins. With SEC-MALLS, it is now possible to measure the M_w distributions of these two protein fractions.

To compare these two fractions, a sequential extraction scheme was employed. Soluble proteins were extracted with two 5-min extractions and insoluble proteins were subsequently extracted with a 24-hr extraction. The only visible difference in the M_w distribution curves was that the material in the exclusion area had a higher average M_w for the insoluble proteins (Fig. 12). For the soluble proteins, the average M_w of the excluded material (S_E) was 3.4×10^7 Da, while that of the insoluble proteins was 8.1×10^7 Da. Again, caution must be exercised when placing the peak markers for the insoluble proteins. Due to the lower levels of these proteins, the DRI signal becomes increasingly low after the main body of the peak. Improper or inaccurate placing of the peak markers in this region will produce unreliable concentration amounts, which can influence the M_w reported.

From this data, it appears that the SDS soluble polymeric proteins range from 3.4×10^7 Da to $\approx 100,000$ Da, while the SDS insoluble proteins ranged up to 8.1×10^7 Da. Note that the M_w measurements in the exclusion limits showed a high degree of variability and increases over time. The reason for this is currently unknown. The numbers reported above represent M_w measurements taken immediately after extraction. These data should be regarded as preliminary until more research can be completed on the variability noted in these measurements, and a greater number of samples can be analyzed to assess the genetic and environmental influence on the M_w distribution as measured by SEC-MALLS.

CONCLUSIONS

The molecular weight distribution of gluten proteins has been correlated to end-use functionality in many studies, often based on the concept of differential solubility. With the development of MALLS for the characterization of gluten proteins, it is now possible to begin to study the actual M_w of the soluble and insoluble polymeric proteins. However, while MALLS produces absolute M_w measurements without the need for calibration, caution must be exercised to ensure that these measurements are accurate. Several factors must be correctly addressed during extraction, separation, and data analysis. In addition, due to the large size of the gluten polymeric proteins, much of the material is eluted at the exclusion limit of the columns, limiting the MALLS data to only the average M_w for this material; the upper size limit cannot be accurately judged from this material. In addition, the inability to solubilize 100% of the protein in an unaltered state also limits the use to characterizing those proteins that can be extracted. Nevertheless, MALLS should prove to be a useful tool for characterizing cereal polymeric proteins.

ACKNOWLEDGMENTS

We would like to thank J. Bietz, R. Myers, K. Preston, and F. MacRitchie for providing useful comments and suggestions to improve this manuscript. Likewise, we wish to thank S. Chandra for assistance in sample preparation and R. Lyne for providing the nitrogen combustion analysis.

LITERATURE CITED

- Astafieva, I. V., Eberlein, G. A., and Wang, Y. J. 1996. Absolute on-line molecular mass analysis of basic fibroblast growth factor and its multimers by reversed-phase liquid chromatography with multi-angle laser light scattering detection. *J. Chromatogr. A* 740:215-229.
- Barth, H. G., and Carlin, F. J., Jr. 1984. A review of polymer shear degradation in size-exclusion chromatography. *J. Liq. Chromatogr.* 7:1717-1738.
- Batey, I. L., Gupta, R. B., and MacRitchie, F. 1991. Use of size-exclusion high-performance liquid chromatography in the study of wheat flour proteins: An improved chromatographic procedure. *Cereal Chem.* 68:207-209.
- Bean, S. R., and Lookhart, G. L. 1998. Faster capillary electrophoresis separations of wheat proteins through modification to buffer composition and sample handling. *Electrophoresis* 19:3190-3198.
- Bean, S. R., Lyne, R. K., Tilley, K. A., Chung, O. K., and Lookhart, G. L. 1998. A rapid method for quantization of insoluble polymeric proteins in flour. *Cereal Chem.* 75:374-379.
- Bean, S. R., Hicks, C., Tuinstra, M., and Lookhart, G. L. 2000. Use of SDS to extract sorghum and maize proteins for free zone capillary electrophoresis (FZCE) analysis. *Cereal Chem.* 78:84-87.
- Bietz, J. A. 1984. Analysis of wheat gluten proteins by high-performance liquid chromatography. I. Baker's Dig. 58(1):15-17, 20-21, 32.
- Bietz, J. A., and Wall, J. S. 1972. Wheat glutenin subunits: Molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Cereal Chem.* 49:416-430.
- Burchard, W., and Cowie, J. M. G. 1972. Selected topics in biopolymeric systems. Pages 725-787 in: *Light Scattering from Polymer Solutions*. M. B. Huglin, ed. Academic Press: London.
- Chakraborty, K., and Khan, K. 1988. Biochemical and breadmaking properties of wheat protein components. II. Reconstitution baking studies of protein fractions from various isolation procedures. *Cereal Chem.* 65:340-344.
- Dachkevitch, T., and Autran, J. C. 1989. Prediction of baking quality of bread wheats in breeding programs by size-exclusion high-performance liquid chromatography. *Cereal Chem.* 66:448-456.
- Danno, G. 1981. Extraction of unreduced glutenin from wheat flour with sodium dodecyl sulfate. *Cereal Chem.* 58:311-313.
- Eisenberg, H. 1976. Biological macromolecules and polyelectrolytes in solution. Pages 48-63 in: *Partial Volumes, Density, and Refractive-Index Increments*. Clarendon Press: Oxford.
- Folta-Stogniew, E., and Williams, K. R. 1999. Determination of molecular masses of proteins in solution: implementation of an HPLC size exclusion chromatography and laser light scattering service in a core laboratory. *J. Biomol. Technol.* 10:51-63.
- Gupta, R. B., Khan, K., and MacRitchie, F. 1993. Biochemical basis of flour properties in bread wheats. I. Effects of variation in the quantity and size distribution of polymeric protein. *J. Cereal Sci.* 18:23-41.
- He, H., Feng, G. H., and Hoseney, R. C. 1991. Differences between flours in the rate of wheat protein solubility. *Cereal Chem.* 68:641-644.
- Huglin, M. B. 1972. Specific refractive index increments. Pages 165-331 in: *Light scattering from polymer solutions*. Academic Press: London.
- Jeng, L., and Blake, S. T. 1993. Evaluation of light scattering detectors for size exclusion chromatography. II. Light scattering equation selection. *J. Appl. Polym. Sci.* 49:1135-1374.
- Jumel, K., Fiebrig, I., and Harding, S. E. 1996. Rapid size distribution and purity analysis of gastric mucus glycoproteins by size exclusion chromatography/multi angle laser light scattering. *Int. J. Biol. Macromol.* 18:133-139.
- Kameyama, K., Nakae, T., and Takagi, T. 1982. Estimation of molecular weights of membrane proteins in the presence of SDS by low-angle laser light scattering combined with high-performance porous silica gel chromatography. *Biochim. Biophys. Acta* 706:19-26.
- Larroque, O. R., and Bekes, F. 2000. Rapid size-exclusion chromatography analysis of molecular size distribution for wheat endosperm protein. *Cereal Chem.* 77:451-453.
- Larroque, O. R., Gianibelli, M. C., Batey, I. L., and MacRitchie, F. 1997. Electrophoretic characterization of fractions collected from gluten protein extracts subjected to size-exclusion high-performance liquid chromatography. *Electrophoresis* 18:1064-1067.
- Lookhart, G. L. 1997. New methods helping to solve the gluten puzzle. *Cereal Foods World* 42:16-19.
- Lookhart, G. L., and Bean, S. R. 2000. Cereal Proteins: Composition of their major fractions and methods for identification. Pages 361-381 in: *Handbook of Cereal Science and Technology*. K. Kulp and J. Ponte, Jr. eds. Marcel Dekker: New York.
- MacRitchie, F. 1975. Mechanical degradation of gluten proteins during high-speed mixing of doughs. *J. Polym. Sci. Symp.* 49:85-90.
- MacRitchie, F. 1992. Physicochemical properties of wheat proteins in relation to functionality. *Adv. Food Nutr. Res.* 36:1-87.
- Meredith, O. B., and Wren, J. J. 1966. Determination of molecular-weight distribution in wheat-flour proteins by extraction and gel filtration in a dissociating medium. *Cereal Chem.* 43:169-186.
- Mhatre, R., Krull, I. S., and Stuting, H. H. 1990. Determination of biopolymer (protein) molecular weights by gradient elution, reversed-phase high-performance liquid chromatography with low-angle laser light scattering detection. *J. Chromatogr.* 502:21-46.
- Mhatre, R., and Krull, I. S. 1993. Determination of on-line differential refractive index and molecular weight via gradient HPLC interfaced with low-angle laser light scattering, ultraviolet, and refractive index detection. *Anal. Chem.* 65:283-286.
- Miyake, J., and Takagi, T. 1981. A low angle laser light scattering study of the association behaviour of a major membrane protein of *Rhodospirillum rubrum* chromatophore at various concentrations of sodium dodecyl sulfate where polypeptides derived from water-soluble globular proteins are solubilized monomerically. *Biochim. Biophys. Acta* 668:290-298.
- Mori, S., and Barth, H. G. 1999. Size exclusion chromatography. Pages 115-129 in: *Molecular Weight Sensitive Detectors*. Springer: Berlin.
- Nagasawa, M., and Takahashi, A. 1972. Light scattering from polyelectrolyte solutions. Pages 671-723 in: *Light Scattering from Polymer Solutions*. M. B. Huglin, ed. Academic Press: London.
- Orth, R. A., and Bushuk, W. 1972. A comparative study of the proteins of wheats of diverse baking qualities. *Cereal Chem.* 49:268-275.
- Potschka, N. 1988. Size-exclusion chromatography of polyelectrolytes: experimental evidence for a general mechanism. *J. Chromatogr.* 441:239-260.
- Reynolds, J. A., and Tamford, C. 1970. Binding of dodecylsulfate to proteins at high binding ratios. Possible implications for the state of proteins in biological membranes. *Proc. Natl. Acad. Sci.* 66:1002-1003.
- Robertson, T. B., and Greaves, J. E. 1911. On the refractive indices of solutions of certain proteins. V. Gliadin. *J. Biol. Chem.* 9:181-184.
- Samsø, M., Daban, J.-R., Hansen, S., and Jones, G. R. 1995. Evidence for sodium dodecyl sulfate/protein complexes adopting a necklace structure. *Eur. J. Biochem.* 232:818-824.
- Sapirstein, H. D., and Fu, B. X. 1998. Intercultivar variation in the quantity of monomeric proteins, soluble and insoluble glutenin, and residue protein in wheat flour and relationships to breadmaking quality. *Cereal Chem.* 75:500-507.
- Sgrulletta, D., and De Stefanis, E. 1989. Relationship between pasta cooking quality and acetic acid-insoluble protein of semolina. *J. Cereal Sci.* 9:217-220.
- Singh, N. K., Donovan, G. R., Batey, I. L., and MacRitchie, F. 1990. Use of sonication and size-exclusion HPLC in the study of wheat flour proteins. I. Dissolution of total proteins in unreduced form. *Cereal Chem.* 67:150-161.
- Southan, M., and MacRitchie, F. 1999. Molecular weight distribution of wheat proteins. *Cereal Chem.* 76:827-836.
- Strazielle, C. 1972. Light scattering in mixed solvents. Pages 633-669 in: *Light Scattering from Polymer Solutions*. M. B. Huglin, ed. Academic Press: London.
- Takagi, T. 1980. Assessment study on the use of the low angle laser light scattering technique for the estimation of molecular weight of the polypeptide forming a complex with sodium dodecyl sulfate. *Biochim. Biophys. Acta* 626:5-14.
- Takagi, T. 1981. Confirmation of molecular weight of *Aspergillus oryzae* α -amylase using the low angle laser light scattering technique in combination with high pressure silica gel chromatography. *J. Biochem.* 89:363-368.
- van Dijk, J. A. P. P., and Smit, J. A. M. 2000. Size-exclusion chromatography-multiangle laser light scattering analysis of β -lactoglobulin and bovine serum albumin in aqueous solution with added salt. *J. Chromatogr. A* 867:105-112.
- Weegels, P. L., Flissebaalje, T., and Hamer, R. J. 1994. Factors affecting

- the extractability of the glutenin macropolymer. *Cereal Chem.* 71:308-309.
- Wen, J., Arakawa, T., and Philo, J. S. 1996. Size-exclusion chromatography with on-line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. *Anal. Biochem.* 240:155-166.
- Wrigley, C. W. 1996. Giant proteins with flour power. *Nature* 381:738-739.
- Wrigley, C. W., and Bietz, J. A. 1988. Proteins and amino acids. Pages 159-275 in: *Wheat Chemistry and Technology*. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.
- Wyatt, P. J. 1992. Combined differential light scattering with various liquid chromatography separation techniques. Pages 35-58 in: *Laser Light Scattering in Biochemistry*. S. E. Harding, D. B. Sattelle, and V. A. Bloomfield, eds. R. Soc. Chem.: Cambridge.
- Wyatt, P. J. 1993. Light scattering and the absolute characterization of macromolecules. *Anal. Chim. Acta* 272:1-40.
- Wyatt Technology Corporation. 1997. *Astra for Windows User's Guide*. Wyatt Technology Corporation: Santa Barbara, CA.
- Zhu, H., Ownby, D. W., Riggs, C. K., Nolasco, N. J., Stoops, J. K., and Riggs, A. F. 1996. Assembly of the gigantic hemoglobin of the earthworm *Lumbricus terrestris*. *J. Biol. Chem.* 271:30007-30021.

[Received December 29, 2000. Accepted April 10, 2001.]