

High Performance Liquid Chromatography: How Proteins Look in Cereals¹

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The fractionation and characterization of cereal proteins may be among the most difficult problems in biochemistry: these proteins are heterogeneous, have unusual solubility characteristics, and have marked tendencies to aggregate, both covalently and noncovalently. Recent reviews (Bietz and Huebner 1980, Kasarda et al 1976, Khan and Bushuk 1979, Mifflin and Shewry 1979, Wall 1979) demonstrate that progress has been made in our understanding of cereal proteins, particularly the wheat endosperm storage proteins, gliadin, and glutenin. Nevertheless, much remains to be known about these proteins, and there is a continuing need for improved methods of separation and analysis.

Cereal protein classification still follows fairly closely that proposed by Osborne (1907). Albumins and globulins are extractable by water or dilute salt solutions, respectively. These classes include enzymes and other proteins necessary for normal physiological processes. Prolamins (gliadin in wheat, zein in corn) are those proteins soluble in aqueous solutions of alcohols (e.g., 70% ethanol or 55% isopropanol). Their solubilities result primarily from their unusual amino acid compositions (rich in glutamine, proline, and hydrophobic amino acids). Most prolamins are monomers of molecular weight (MW) 30,000–40,000. Prolamins have no known function other than storage and are very heterogeneous because of duplication and subsequent nonlethal mutation of genes; as a result, much homology exists among prolamins (Bietz et al 1977). In addition, wheat polyploidy further increases gliadin heterogeneity.

If all albumins, globulins, and prolamins are extracted from a cereal, the remaining endosperm protein can be regarded as glutelins. Such a fractionation is, however, complicated by the tendency for cereal proteins to associate: for example, gliadin strongly binds to glutenin (Bietz and Wall 1975), the glutelin fraction of wheat. Glutelins are high-MW polymers consisting of subunits joined both through covalent (disulfide) and noncovalent (primarily hydrogen and hydrophobic) bonds. They are soluble, if at all, only in dilute acids, alkali, high concentrations of denaturants (such as urea or guanidine hydrochloride), or in solutions containing detergents (such as sodium dodecyl sulfate [SDS]). To fully solubilize glutelins, it is frequently necessary to cleave and stabilize disulfide bonds through reduction and alkylation and to disrupt noncovalent bonds with denaturants and detergents. Glutelin subunits are then soluble and more easily characterized; some are similar to prolamins, but in many cereals, such as wheat and corn, glutelin subunits and prolamins have uniquely different structures. Other glutelin subunits resemble albumins and globulins, whereas some, such as the high-MW subunits of wheat glutenin, occur in no other protein class.

Numerous reasons exist to isolate and characterize cereal proteins. Amounts or types of proteins may directly influence functional properties; for example, native glutenin MW relates closely to wheat breadmaking quality (Orth and Bushuk 1972, Huebner and Wall 1976), as does the presence of specific high-MW glutenin subunits (Payne et al 1980). If the identity of these quality-

promoting polypeptides can be established, it may be possible to breed improved varieties by selecting for specific proteins as "markers" of quality. Similarly, nutritionally important proteins could be bred into new varieties. Geneticists may also use such information to detect specific chromosomes, such as those introduced from alien species to impart desirable characteristics, and to determine genetic interrelationships of lines. Finally, knowledge of cereal proteins and of their properties can lead to valuable methods for identification, classification, and quality control.

Relatively few methods have actually been used to isolate and characterize cereal proteins (Bietz 1979). Electrophoresis generally separates proteins on the basis of both size and charge; however, in SDS electrophoresis, separations are based only on molecular size, and in isoelectric focusing, only differences in ionized amino acid content influence separations. Electrophoresis techniques can also be combined into two-dimensional methods of high resolving power. In general, however, electrophoresis is of limited value for preparative isolation of cereal proteins.

Chromatographic methods are also valuable for cereal protein isolation and characterization. In ion-exchange chromatography, proteins bound ionically to a support are selectively displaced, through changes in pH or ionic strength, as a function of their binding affinities. In size-exclusion chromatography (also called gel permeation, gel filtration, and molecular exclusion chromatography), proteins separate on the basis of molecular size as they diffuse through a porous matrix. Hydrophobic interaction chromatography separates proteins on the basis of how strongly hydrophobic amino acids on their surfaces interact with nonpolar chromatographic supports (Caldwell 1979, Chung and Pomeranz 1979, Popineau et al 1980, Godon and Popineau 1981). All these electrophoretic and chromatographic methods have been successfully used for cereal proteins, but there is a real need for improved methods. High performance liquid chromatography (HPLC) seemed to have such a potential.

HPLC has for many years been an excellent technique for separating low-MW compounds due to the development of uniform spherical (5–10 μm) silica supports, to which are bonded various stationary phases differing in selectivity. Most such supports have pore sizes of 80–100 \AA , which cannot be penetrated by many proteins. Today, however, silicas having pore sizes of 300 \AA or larger ("large-pore" or "wide-pore" silicas) are available, making HPLC of proteins a reality (Regnier and Gooding 1980, Hearn et al 1982). Proteins having MW of several hundred thousand can penetrate these supports and fully interact with their stationary phases. Columns are also "end-capped" to prevent ionic adsorption of proteins, which largely prevents size-exclusion chromatography of cereal proteins on controlled-pore glass.

Microparticulate silica-based columns having 300–500 \AA pores are now available for reversed-phase, size-exclusion, and ion-exchange separations (Wehr 1984). Reversed-phase HPLC (RP-HPLC) separations are eliciting the greatest interest among protein chemists, because proteins are resolved on the basis of differences in surface hydrophobicity, a characteristic largely independent of MW and charge. RP-HPLC therefore complements most other electrophoretic and chromatographic procedures. RP-HPLC is analogous to hydrophobic interaction chromatography, but it has significantly better resolution and speed.

HPLC is now also becoming widely applied to cereal proteins. This paper reviews progress to date in applying reversed-phase, size-exclusion, and ion-exchange HPLC to cereal proteins, gives specific examples and applications of these methods, and demonstrates their significant advantages.

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MATERIALS AND METHODS

Details of most HPLC procedures have been described previously (Bietz 1983, Bietz 1984a,b); this section therefore reviews the topic on a more general basis, emphasizing only major points.

Apparatus

Most HPLC apparatus used today, whether purchased as a complete system or assembled from individual components, is reliable and suitable for analysis of cereal proteins. Pumps deliver solvents at constant flow rates, giving good run-to-run reproducibility. Size-exclusion HPLC (SE-HPLC) separations of proteins are run isocratically with one pump, but RP-HPLC and ion-exchange HPLC (IE-HPLC) require gradients to elute bound proteins. Gradients can be generated either by using two pumps having outputs controlled by a gradient programmer or by using one pump that delivers a gradient generated before the pump inlet. Both methods are acceptable and give comparable results.

The detector most useful for cereal protein HPLC is a variable wavelength ultraviolet (UV) detector. Proteins can be detected either at 280 nm, on the basis of tryptophan absorption, or at 210–225 nm, on the basis of peptide bonds. All proteins can be detected at 210 nm, which is approximately 100 times more sensitive than 280 nm; however, many nonprotein materials also absorb at 210 nm, somewhat limiting the solvents that can be used.

An important, though not essential, component of an HPLC system is an automatic sample injector. Although inexpensive syringe-filled loop injectors may also be used, an automatic injector greatly increases productivity and decreases operator time. Samples can be repeatedly analyzed, injection volumes can be varied, and analyses can be performed at night or during weekends.

HPLC equipment can be fairly expensive, but it should be noted that the same equipment used for HPLC analyses of low-MW compounds can also be used for proteins. Since HPLC equipment is now common in many laboratories, only a different column may be necessary to begin protein analysis.

Columns

HPLC columns suitable for proteins have larger pore sizes than most standard HPLC columns (300–500 Å vs. 80–100 Å). Prices for most HPLC columns are similar (\$200–400), but SE-HPLC columns may cost considerably more. Column stability is generally very good, however, so cost per analysis is low. Typically, hundreds or thousands of analyses can be performed on a column before performance noticeably deteriorates. However, a guard column is essential for column longevity; it acts both as a final filter and to

adsorb materials that may strongly bind to the support. If suitable guard column packings are not available, an in-line filter (0.2–0.45 μm) should precede the column. Even with such procedures, some materials may accumulate on a column; usually, washing with stronger organic solvents, urea solutions, or dimethyl sulfoxide elutes such materials and restores column performance.

Numerous columns suitable for protein HPLC are now available (Hearn et al 1982; Wehr 1984), and additional column types are frequently introduced. For example, large-pore HPLC columns are now available from Baker, Beckman, Bio-Rad, Brownlee, ES Industries, Separations Group, Supelco, SynChrom, Varian, and Whatman; available bonded phases include C_{18} , C_8 , C_4 , C_3 , cyano, phenyl, diphenyl, and fluoropropyl, in various pore and particle sizes.

Data Handling

For some applications, direct examination of chromatographic tracings may answer specific questions. Frequently, however, further processing of chromatographic data is desirable. This can be accomplished using fairly simple integrators that provide accurate elution times and peak areas. For many applications, however, it is desirable to save raw analog data on tape or disk; data subsequently can be restored for recalculation, for replotting to a different scale (for example, to visualize minor components), or for comparison plots using a computer. When raw chromatographic data are saved, it is also possible to subtract baselines from data and to analyze hybrids and mixtures through summation or difference plots of individual chromatograms.

Reagents and Solvents

Components of HPLC solvents must be of high purity to permit monitoring of the column effluent at approximately 210 nm and to avoid spurious results. Similarly, the water should be of very low conductivity and free of organic compounds, which lead to peaks in blank runs. Such water is obtained by passing distilled water through a system containing mixed-bed deionization cartridges and a carbon filter.

The solvent system most frequently used for RP-HPLC of proteins employs acetonitrile (ACN), water, and trifluoroacetic acid (TFA); as solvent hydrophobicity increases during the gradient, bound proteins are selectively displaced. For silica supports, solvent pH must be maintained between 2 and 8. All solvents must be filtered (0.45 μm , nylon), and further deaeration using vacuum or sonication may be necessary to prevent degassing in the detector flowcell. Solvents are prepared and changed frequently to prevent contamination and ACN concentration changes due to evaporation.

Samples

Samples for analysis can be dissolved in nearly any solvent and at any suitable concentration, because after injection, proteins bind to the head of the column and equilibrate with column solvents. Sample pH should be 2–8, the same as for solvents; and excessive acidity should be avoided to prevent deamidation. Most importantly, all samples should be filtered (0.45 μm , possible even for small samples through the use of centrifugal filtration apparatus) or centrifuged before analysis to remove particulates. Many sample solutions remain stable indefinitely, but it is always necessary to look for evidence of precipitation through association after storage.

RP-HPLC OF CEREAL PROTEINS

Initial Trials

While attempting to use RP-HPLC to analyze large peptides (Pearson et al 1981), we found that the same general method, employing an ACN gradient in the presence of TFA, could effectively fractionate intact cereal proteins. One of our first trials of the methods, analyzing a mixture of low-MW gliadins, is shown in Figure 1. This sample, of the type that is usually resolved into 20–30 bands by one-dimensional polyacrylamide gel electrophoresis (PAGE) (Wrigley et al 1982) and a maximum of 46 spots by

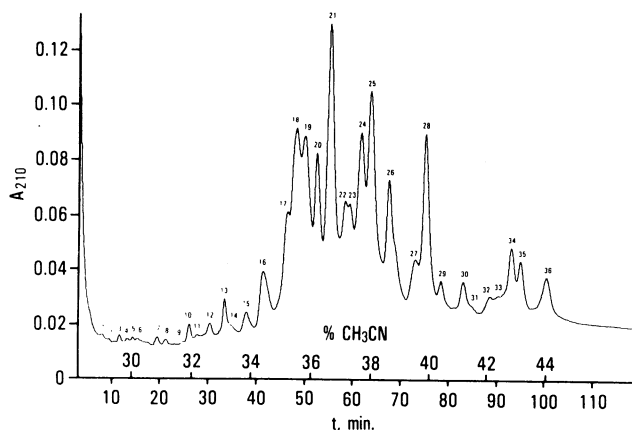


Fig. 1. RP-HPLC separation of reduced and pyridylethylated low-MW gliadin proteins from Ponca wheat. A sample of 0.1 mg in a volume of 100 μl was applied to a SynChropak RP-P (C_{18}) column (250 \times 4.1 mm i.d.) and eluted with a linear gradient from 20 to 50% solvent B during 120 min. The column effluent was monitored at $A_{210} = 0.1$ absorbance units full scale (a.u.f.s.)/10mV. Solvent A, 15% acetonitrile + 0.1% TFA; solvent B, 80% acetonitrile + 0.1% TFA. From Bietz 1983.

two-dimensional PAGE (Wrigley and Shepherd 1973), was here separated into 36 peaks (including unresolved shoulders and minor peaks easily visible upon scale expansion [Bietz 1983]).

Optimization of Conditions

Subsequent studies established optimal conditions for RP-HPLC analyses of cereal proteins and also examined the method (Bietz 1983). Using an organic phase containing ACN and TFA, we found that nearly all cereal proteins were adsorbed at ACN concentrations of 15%, and all proteins were eluted by 80% ACN. We therefore chose 15 and 80% ACN (containing 0.1% TFA) as limiting solvents A and B, from which all solvents of intermediate ACN concentrations could be prepared. Other organic phases (including methanol and 2-propanol) and ion-pairing agents (including hexafluorobutyric acid and SDS) were also tested. Although they generally offer no significant advantage over ACN and TFA, they may in some cases alter column selectivity and be useful in difficult separations.

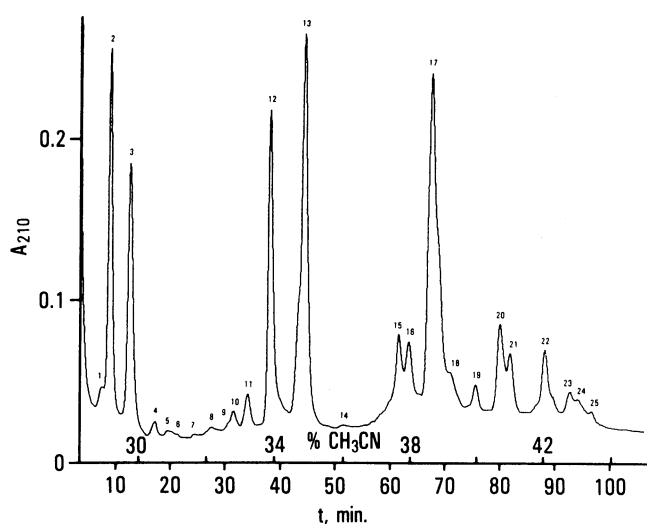


Fig. 2. RP-HPLC separation of reduced and pyridylethylated omega gliadin from Ponca wheat. A sample of 0.1 mg was applied in a volume of 100 μ l and eluted with a linear gradient from 20 to 50% B during 120 min. The column effluent was monitored at $A_{210} = 0.2$ a.u.f.s.; other conditions as in Figure 1. From Bietz 1983.

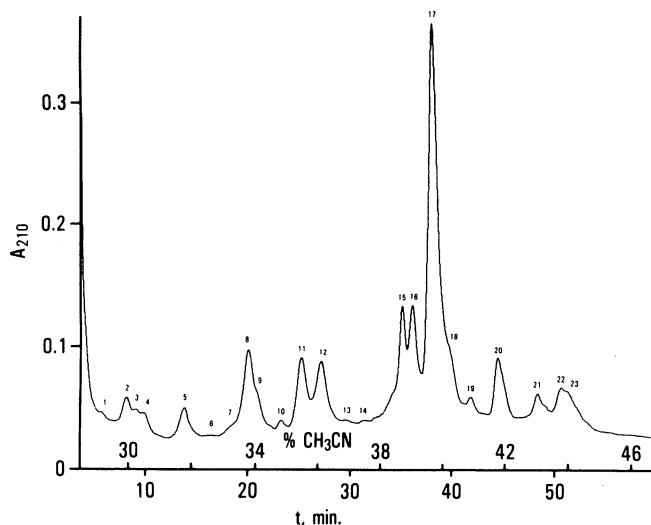


Fig. 3. RP-HPLC separation of pyridylethylated ethanol-soluble reduced glutenin subunits (0.1 mg) from Ponca wheat. The sample was applied in a volume of 100 μ l and eluted with a linear gradient from 20 to 50% B during 60 min. The column effluent was monitored at $A_{210} = 0.2$ a.u.f.s.; other conditions as in Figure 1. From Bietz 1983.

Sample size may vary considerably. For initial studies of heterogeneous fractions, approximately 0.1 mg is usually applied; for purified fractions 1 μ g or less may be used. At the other extreme, 5 mg protein may be applied to an analytical column without significant loss of resolution. Typical sample volumes are 20–100 μ l, but samples of several milliliters may be applied if initial conditions prevent protein elution until a gradient is initiated.

Optimal gradients for unknown samples are generally determined by first using a wide gradient (0–100% solvent B) with a slope of approximately 2% B/min. From calculated ACN concentrations corresponding to minimum and maximum elution times, a linear gradient spanning the desired hydrophobicity range is devised; run times are then chosen to provide a gradient slope of approximately 0.5% B/min (e.g., 20–50% B/60 min). Most analytical runs are performed at 1.0 ml/min at room temperature; constant temperature is necessary to maximize reproducibility. Recent results (Bietz and Cobb, 1984), however, demonstrate that very rapid (10–15 min) gradients using a flow rate of 3 ml/min,

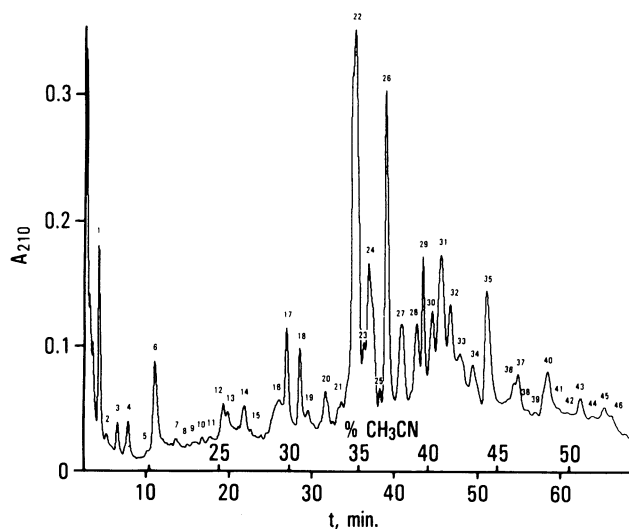


Fig. 4. RP-HPLC separation of proteins extracted from a single kernel of the wheat variety Chinese Spring with 0.1M NaCl. A sample of 100 μ l was applied to the column and eluted with a linear gradient from 0 to 55% B during 60 min; the column effluent was monitored at $A_{210} = 0.2$ a.u.f.s.; other conditions as in Figure 1. From Bietz 1983.

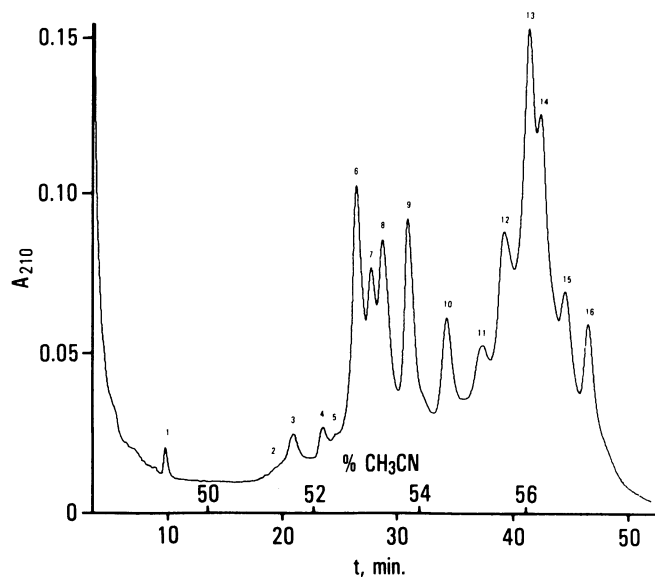


Fig. 5. RP-HPLC separation of reduced and pyridylethylated zein (0.1 mg applied in 100 μ l). The chromatogram was developed with a linear gradient from 50 to 70% B during 60 min, and monitored at $A_{210} = 0.2$ a.u.f.s.; other conditions as in Figure 1. From Bietz 1983.

combined with elevated column temperature (70°C, which provides increased resolution), also provide good resolution combined with greatly increased speed. Separations are monitored at 210 nm, using a detector sensitivity of 0.2–0.4 absorbance units full scale to a 10 mV recorder. For preparative runs, 210 nm may be too sensitive, and 254 or 280 nm is used.

Characteristics of RP-HPLC Separations

The above HPLC conditions give highly reproducible separations. The relative standard deviation for elution times from replicate runs averaged 0.3295. Typically, elution times for the same proteins differ by no more than 0.10–0.15 min between runs, and replicate chromatograms are visually superimposable. Since elution times depend primarily on ACN concentration, uniform solvent preparation is necessary to ensure identical results. Consequently, it is highly desirable to analyze a heterogeneous reference sample periodically to monitor solvent compositions and detect problems. Alternatively, it may be desirable to use water and ACN (each containing 0.1% TFA) as limiting solvents to prevent evaporative changes in composition, and to use an aliphatic series of non-protein compounds as internal or external standards to monitor column performance (Bietz and Cobb, 1984). Similarly, blanks should be analyzed to determine impurities in the water or the organic solvents. Before samples are analyzed, the column should be washed with a solvent more hydrophobic than that used in the gradient (e.g., 100% ACN or 100% solvent B) to remove any tightly bound proteinaceous material. Decreased resolution, as judged by separations of a reference sample, or increased operating pressure may indicate tightly bound or insoluble protein, carbohydrate, or lipid on the column. Regeneration with solutions such as 2-propanol, urea, dimethylsulfoxide, or chloroform (taking care to ensure miscibility of solvents at each solvent change) usually restores much-used columns to good condition. With such care, a column can provide hundreds or even thousands of analyses.

RP-HPLC Separations of Other Protein Classes

RP-HPLC is useful for analysis of all classes of wheat proteins and for proteins of other cereals (Figs. 2–5). A separation of a wheat protein fraction enriched in omega-gliadins is shown in

Figure 2. Omega gliadins have MW of 65,000–80,000, contain no cysteine, and differ significantly from other gliadins (Fig. 1) in amino acid composition. RP-HPLC separated this mixture into at least 25 components; some were similar to low-MW gliadin (Fig. 1), but four major omega gliadin polypeptides elute much earlier, apparently due to their reduced surface hydrophobicities.

RP-HPLC can also analyze wheat glutenin subunits obtained upon reduction of disulfide bonds and alkylation of resulting cysteine residues (Fig. 3). Ethanol-soluble glutenin subunits were resolved into approximately 23 components, mostly different from those in other wheat gluten classes (Figs. 1 and 2). The maximum protein size suitable for wide-pore RP-HPLC analysis has not been strictly established, but theoretically it should be several hundred thousand MW; thus, RP-HPLC is suitable for any single-chained polypeptide, including the largest glutenin subunits of approximately 130,000 MW (Bietz and Wall 1972).

This has recently been confirmed by Burnouf and Bietz (1984b). Optimal conditions were determined for extraction, reduction, alkylation, and RP-HPLC analysis of all glutenin subunits, including those of high MW. High-MW subunits characteristically elute before lower-MW ethanol-soluble subunits; varietal differences among these subunits indicate allelic variation, which may relate to breadmaking quality.

RP-HPLC can also be used to characterize heterogeneous mixtures of wheat albumins and globulins obtained by 0.1 M NaCl extraction (Fig. 4). This complex chromatogram contains approximately 46 major components, plus at least 40 more visible upon scale expansion; frequently more than 100 peaks (counting minor ones and unresolved shoulders) are resolved in such samples.

The sample analyzed in Figure 4 differs from the others (Figs. 1–3) in that it was directly extracted from a single wheat kernel. Analysis of only a small percent of the resulting centrifuged extract produced this chromatogram, indicating the high sensitivity of the method. Similar protein extractions from single kernels are also being used extensively for other protein types (see below).

RP-HPLC separations are not limited to wheat proteins. Figure 5 shows an RP-HPLC separation of zein, the prolamins of corn. Zeins have significantly higher surface hydrophobicity than most wheat proteins (Figs. 1–4), as indicated by the more

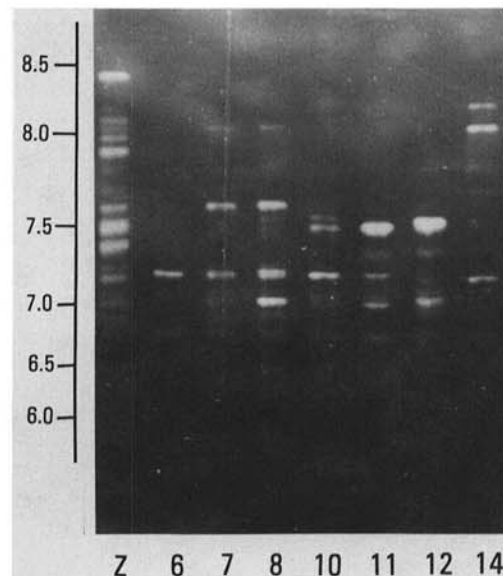
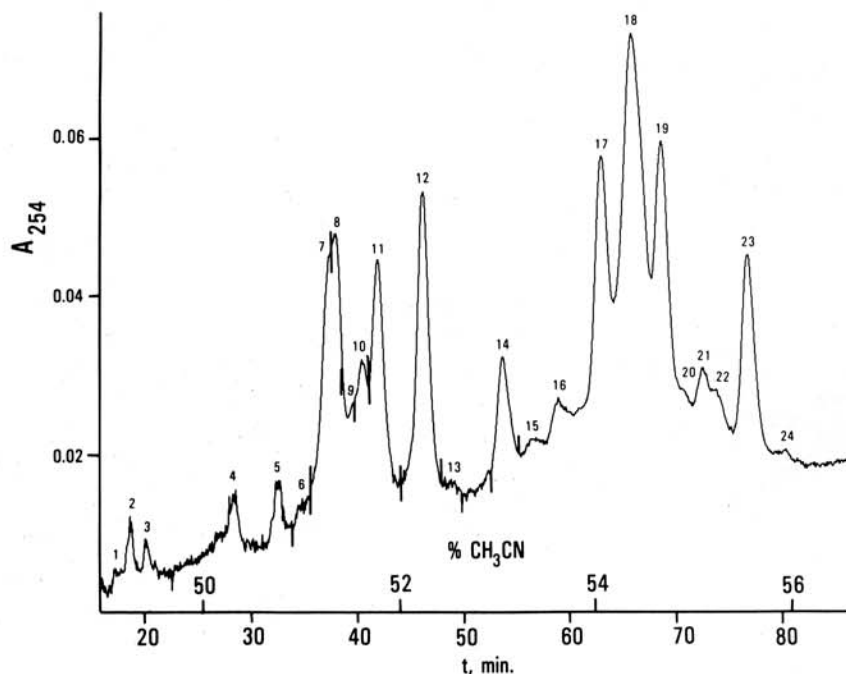


Fig. 6. (left) RP-HPLC separation of 5.0 mg of reduced and pyridylethylated zein on SynChropak RP-P (250 × 4.1 mm). Sample (1.0 ml of a 5 mg/ml solution in 50% solvent A [15% acetonitrile + 0.1% TFA] + 50% glacial acetic acid) was applied to the column equilibrated with 20% solvent B (80% acetonitrile + 0.1% TFA) + 80% solvent A, and then eluted with a linear gradient from 50 to 70% B during 120 min. The column effluent was monitored at $A_{254} = 0.4$ a.u.f.s./10mV. (right) Isoelectric focusing of PE-zein (Z) and fractions obtained by RP-HPLC (from left); approximately 25% of each collected fraction was analyzed. Isoelectric points are indicated at the left-hand margin. From Bietz 1983.

hydrophobic gradient conditions required to elute all components (50–70% B, as compared to 20–50% B for most wheat proteins). These results agree with the abundance of hydrophobic amino acids in zein, and with zein's limited solubility except in the presence of denaturants, detergents, or high concentrations of organic solvents. RP-HPLC resolved approximately 16 peaks from reduced and alkylated zein; similar results can be obtained for native zein. Elevated column temperature may particularly improve its resolution (Bietz and Cobb 1984). In general, RP-HPLC resolves as many or more components from most protein mixtures than most other chromatographic or electrophoretic methods.

Preparative RP-HPLC

RP-HPLC can also be used to isolate purified fractions in sufficient amounts for further studies (Bietz 1983). In Figure 6a, 5 mg of pyridylethylated zein was fractionated on an analytical column and subsequently characterized by isoelectric focusing (Fig. 6b). Significant differences occurred between even closely eluting fractions. Sample heterogeneity may prevent RP-HPLC from isolating components in a single chromatographic run, although significant purification occurs. Because there is no clear relationship between surface hydrophobicity (as indicated by elution volume) and ionic character (as indicated by isoelectric points), RP-HPLC, used in conjunction with other chromatographic or electrophoretic techniques, should be able to isolate significant amounts of highly purified proteins.

Preparative RP-HPLC has been used to purify cereal proteins (Burnouf and Bietz 1984a). In this study, a preparative (250 × 10 mm i.d.) column fractionated 20–25 mg of protein per run, and nearly homogeneous durum gliadin proteins (corresponding to PAGE bands 42 and 45, which correlate to durum gluten strength and weakness, respectively) were isolated in a single step (Fig. 7).

Preparative RP-HPLC has also been combined with gel filtration and ion-exchange chromatography to isolate gliadin proteins (Huebner and Bietz 1984). Gliadins were first fractionated according to MW on Sephadex G-100, and low-MW gliadins were then chromatographed on sulfoethyl cellulose (SEC). Finally, fractions from the SEC column were subjected to preparative RP-HPLC. Several gliadins were obtained in a good state of purity, as shown by gel electrophoresis and isoelectric focusing.

Identification of Wheat Varieties

Wheat gluten proteins are very heterogeneous, and their synthesis does not vary under a wide range of conditions. Mutations have led to significant variation among gluten proteins, making them accurate genotypic indicators. Gliadin proteins, in particular, have been extensively analyzed by electrophoresis for varietal identification (Wrigley et al 1982). Because resolution of gliadins by RP-HPLC is generally at least as good as that by one-dimensional electrophoresis (Fig. 1), we attempted to use RP-HPLC for identification of cereal varieties.

Initial RP-HPLC analyses of gliadins from numerous wheat varieties revealed considerable variation among their chromatograms. To determine optimal gliadin extraction conditions for RP-HPLC analysis as the basis for varietal identification, Bietz et al (1984a) investigated the effects of extraction time, defatting, prior NaCl extraction, type of extractant, and age of extract on the resulting chromatograms. Optimal extraction was achieved using nondefatted flour, a 30-min extraction time, and no prior NaCl extraction. Aqueous 70% ethanol was the most suitable extractant tested, and extracts were stable even at room temperature for at least 28 days. Thus, very simple extraction conditions can extract gliadin for RP-HPLC analysis; resulting chromatograms can serve to identify wheat varieties.

Using these extraction conditions, Burnouf et al (1983b) and Bietz et al (1984b) examined numerous hard red winter, hard red spring, soft red winter, white, durum, and French wheat varieties. A comparison of five hard red spring wheat varieties is shown in Figure 8. For these analyses, gliadins were extracted from single wheat kernels. RP-HPLC could differentiate and thereby identify these and most other varieties on the basis of numerous

quantitative and qualitative differences (including peaks a–f, Fig. 8). Frequently, major qualitative differences make such differentiation simple, as is the case for Anza, a wheat having reduced breadmaking quality. At other times, however, very similar chromatograms result (as for Era and Solar, which also gave identical electrophoregrams). Nevertheless, minor differences (peaks d–f) may differentiate these two varieties. Such differences are more apparent by RP-HPLC than by electrophoresis. Until the extent of natural variability within a variety is better understood, one should be cautious about identifications based on minor differences. It is also possible to obtain misidentified samples, and most grain samples contain minor amounts of contaminants and biotypes; thus, to establish a standard chromatogram for any variety, it is necessary to analyze either numerous single kernels or (preferably) a bulk sample from more than one source. Single-kernel extracts can then be compared to such standards. Rapid RP-HPLC procedures (Bietz and Cobb 1984), capable of approximately 100 analyses per day, now make determination of varietal purity via single-kernel analysis a real possibility.

RP-HPLC may indicate pedigree and thus could be useful for registration or certification. Analyses of closely related wheat varieties show very similar gliadin RP-HPLC patterns, suggesting the utility of RP-HPLC for determining evolutionary relationships or hybrid parentage. RP-HPLC may also point out misidentified samples: for example, it seems unlikely that Era and Solar, which have very different reported pedigrees (Zeven and Zeven-Hissink 1976), should have gliadins nearly identical by electrophoresis and by RP-HPLC.

We have found that wheat varieties having similar gliadins frequently are easier to differentiate by RP-HPLC than by electrophoresis. This may be due partially to the somewhat better resolution of RP-HPLC, but more importantly, it reflects the complementary nature of the methods. For example, proteins differing only by nonpolar amino acid substitutions may have identical gel filtration, ion exchange, and electrophoresis characteristics, but different hydrophobicities may make them separable by RP-HPLC.

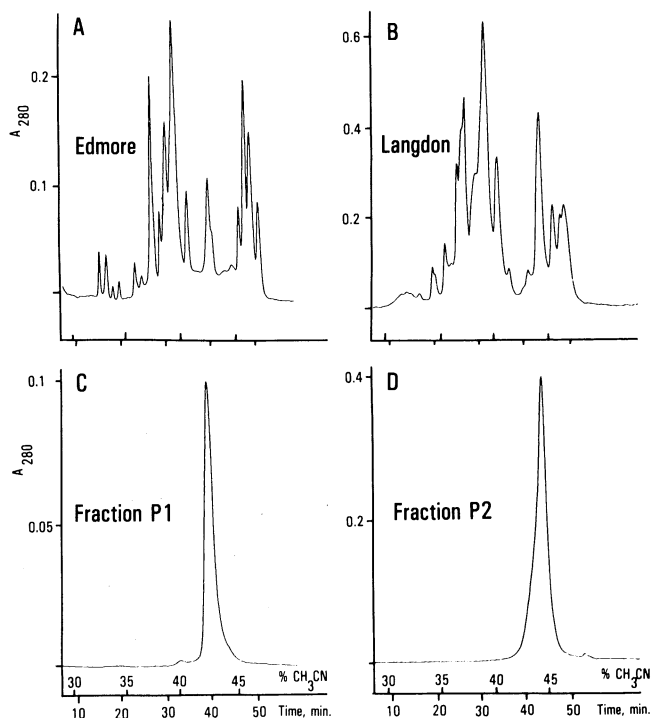


Fig. 7. Preparative RP-HPLC on SynChropak RP-P (250 × 10 mm) of gliadins from durum wheat varieties (A) Edmore and (B) Langdon and of isolated chromatographic fractions. Samples were eluted with a linear gradient from 20% to 55% B during 55 min at 3 ml/min, and the column effluent was monitored at $A_{280} = 0.4$ a.u.f.s./10mV. Solvents A and B were as indicated in Figure 1. From Burnouf and Bietz 1984a.

In general, more samples can be analyzed per day by electrophoresis than by RP-HPLC, and equipment is admittedly more complex and expensive (although the method may be less subject to variability). Therefore, we regard RP-HPLC as an

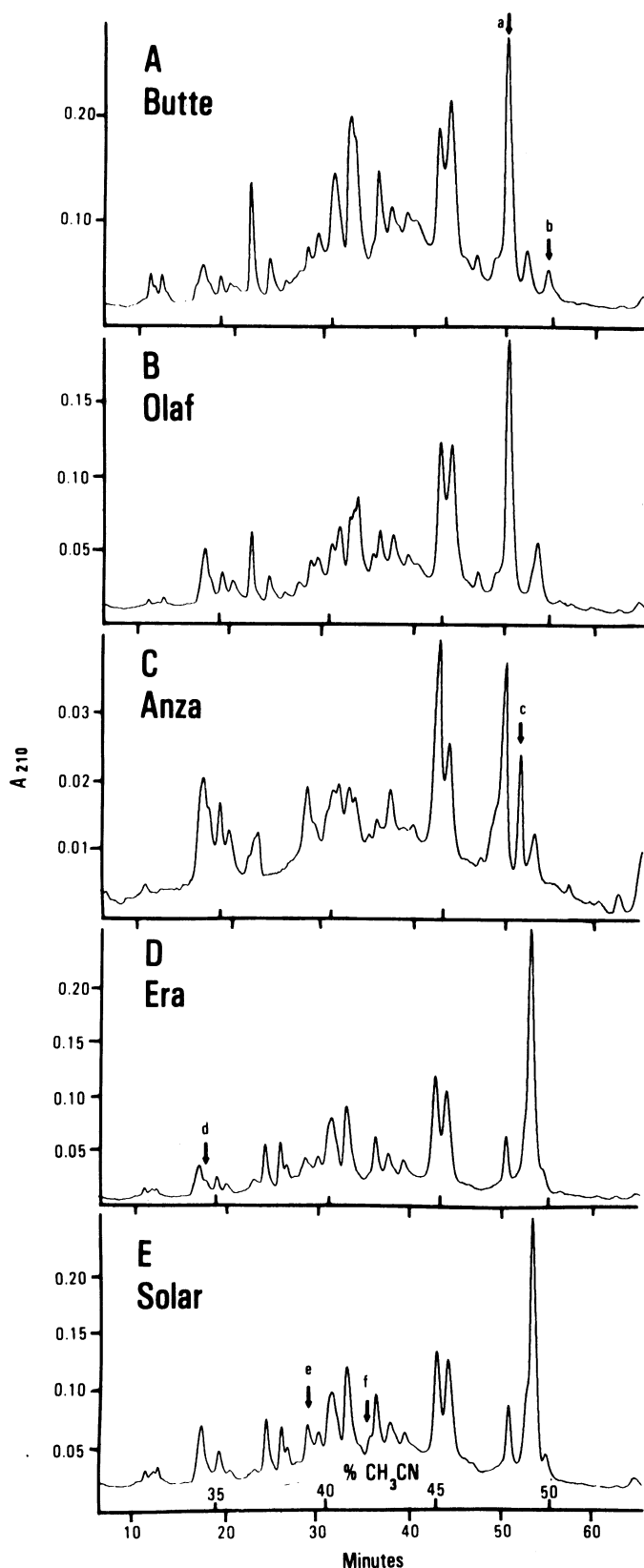


Fig. 8. RP-HPLC comparison of gliadin extracts from the hard red spring wheat varieties (A) Butte, (B) Olaf, (C) Anza, (D) Era, and (E) Solar. Arrows indicate peaks useful in differentiating these varieties. From Bietz et al 1984b.

extremely valuable complementary method having unique advantages. First, it can be automated, and automated varietal identification is a distinct possibility. Secondly, it can differentiate some varieties that other methods cannot. Third, it can accurately quantitate data, which is more difficult in electrophoresis. Fourth, important samples can be characterized quickly. Only about 1.5 hr is required to grind a wheat kernel, extract gliadin proteins, and perform RP-HPLC analysis to achieve maximum resolution; with electrophoresis, 1–2 days may be required to obtain results.

Modification of RP-HPLC methodology for varietal identification (Bietz and Cobb 1984), using rapid gradients, increased flow rates, and elevated column temperature, permits analysis of about 100 samples per day per instrument, with relatively minor loss of resolution. Differentiation of most varieties is still easily possible by RP-HPLC using these conditions, and the number of samples which can be analyzed per day is similar to that possible by electrophoresis.

Another example of using RP-HPLC to characterize wheat samples is shown in Figure 9. If a line is not totally homogeneous, yet is being used in breeding, it seems necessary to identify parental lines used in test crosses. Similarly, breeders may select for variability to identify desirable genotypes. Such may be the case for Nap Hal, a hexaploid wheat that has genes for both elevated protein and lysine contents but is known to be somewhat heterogeneous. RP-HPLC results for four of 12 kernels selected by phenotypic differences (Fig. 9) clearly demonstrate qualitative and quantitative differences among gliadins, showing different genotypes. Thus, RP-HPLC should become a useful selection tool in wheat breeding and improvement.

RP-HPLC Prediction of Pasta Cooking Quality

When durum wheat gliadins are examined by RP-HPLC (Bietz et al 1984b), it is apparent that, in addition to differentiating varieties, RP-HPLC divides them into two groups on the basis of late-eluting components. Representative results of Burnouf and Bietz's (1984a) examination of many durum varieties are shown in Figure 10.

In these four chromatograms, peaks A1–A4 serve to divide durums into two major categories, either containing peaks A1, A2, and A4 (varieties Pelissier and Mondur in Fig. 10; also Edmore, Vic, and Wakooma) or else peak A3 (Leeds and Ward in Fig. 10; also Langdon). This division of durums on the basis of chromatographic peaks is identical to that based on gliadin electrophoresis bands 45 and 42 (Damidaux et al 1978), which predict durum gluten strength and weakness, respectively. Durum gliadins were fractionated by preparative RP-HPLC (Fig. 7), and it was established that peak A3 corresponds to band 42, while peak A2 corresponds to band 45. These results permitted development of an RP-HPLC analytical procedure that differentiates durums according to quality in less than 10 min, permitting approximately 200 analyses per day. The procedure may serve to predict durum quality when large numbers of samples must be analyzed, as in early generations of wheat breeding, and could be used as a routine test for processing quality.

This example uses RP-HPLC to predict quality, but many further applications are possible. For example, Burnouf and Bietz (1984b) have shown that various hexaploid wheat high-MW glutenin polypeptides related to breadmaking quality may be rapidly extracted and identified through RP-HPLC. Similarly, RP-HPLC analysis of hexaploid wheat gliadins related to breadmaking quality should be possible.

Use of RP-HPLC in Genetic Studies

Because polypeptides are coded by specific genes, protein analysis can indicate the genetic composition of cereals, thus assisting plant improvement through traditional breeding or molecular biological approaches. For example, electrophoresis of wheat aneuploids (having atypical chromosomal contents) has located genes coding specific gliadin and glutenin polypeptides. Subsequent analyses can identify quality-promoting proteins or indicate lines having characteristics related to genes closely linked with genes for "marker" proteins.

RP-HPLC may also be used in genetic studies (Fig. 11). Here, analyses of ditelocentric lines of the wheat variety Chinese Spring, which contain the indicated chromosome arms but lack the nondesignated arms, established the chromosomal control of eight gliadins. In additional analyses of all nullisomic-tetrasomic and ditelo lines of Chinese Spring, J. A. Bietz and T. Burnouf (*unpublished data*) determined the coding of all hexaploid wheat gliadins resolved by RP-HPLC. Similarly, Burnouf and Bietz (1984b) established chromosomal control of high-MW glutenin subunits in Chinese Spring, as well as coding of all glutenin subunits resolved by RP-HPLC (*unpublished data*). In other studies, Burnouf and Bietz (*unpublished data*) have established the coding of durum polypeptides through aneuploid analysis and compared diploid, tetraploid, and hexaploid species of *Triticum* and closely related genera to demonstrate and establish genetic relationships. Such information should make RP-HPLC a significant tool for analyzing experimental lines on the basis of polypeptides associated with specific characteristics.

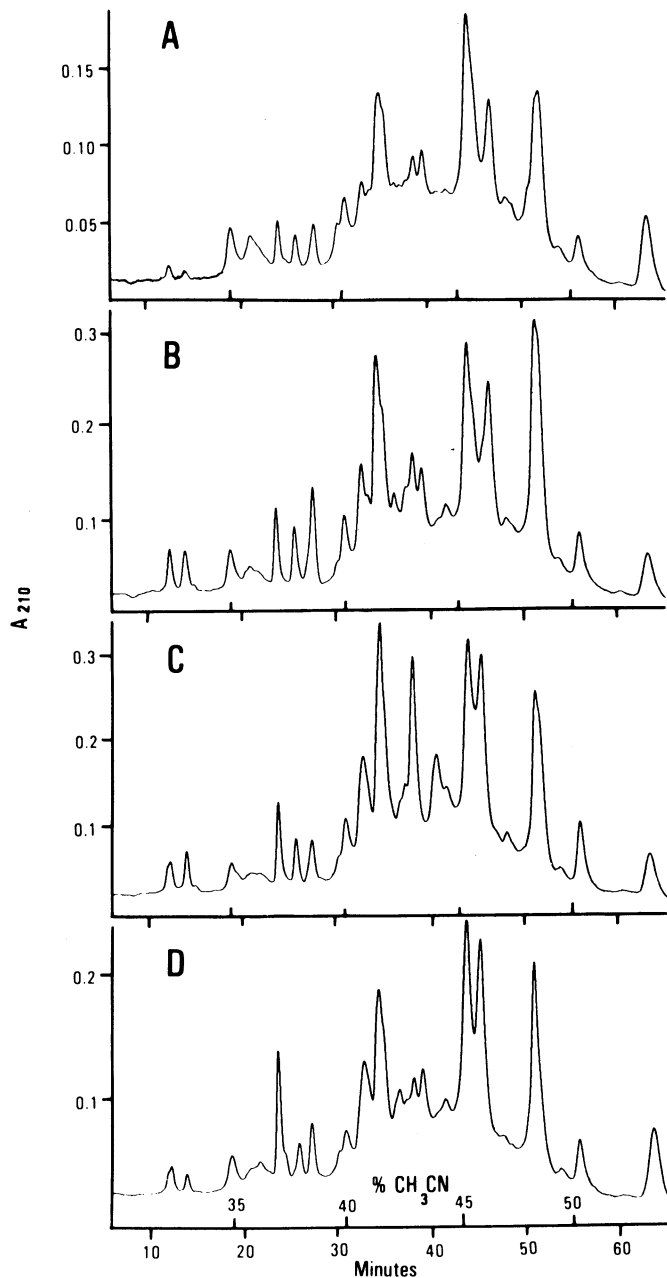


Fig. 9. RP-HPLC analysis of gliadins extracted from four individual kernels of the hexaploid wheat Nap Hal. From Bietz et al 1984b.

Comparison of RP-HPLC Columns

Most of the above studies were performed using one of the first large-pore RP-HPLC column types suitable for protein analysis. Now, however, many such columns are available from numerous suppliers. Although initial separations were very good, we wished to establish which columns give optimal separations of cereal proteins, to determine how much variability exists among columns of the same type, and to establish how columns having different selectivities can be used in combination to isolate proteins. To these ends, Bietz et al (1983) examined numerous columns from different sources and having different bonded phases, using a standard set of protein samples. Typical results for one sample obtained using four different columns are presented in Figure 12.

Most large-pore RP-HPLC columns were suitable for cereal protein analysis, but differences in resolution and selectivity were observed. Columns having the same bonded phases but from different manufacturers were surprisingly similar, and columns from the same source were very similar in performance, suggesting that results from different laboratories should be comparable. The differing selectivities afforded by columns with different bonded phases can be very useful in isolating polypeptides.

RP-HPLC Analysis of Glutenin

Glutenin is the highest MW protein fraction of wheat and is closely related to wheat's elasticity and strength. The lower MW ethanol-soluble subunits of glutenin can be analyzed by RP-HPLC (Fig. 3), but additional methods are necessary to analyze higher MW ethanol-insoluble subunits, which are related to quality (Payne et al 1979, Burnouf and Bouriquet 1980). Burnouf and Bietz (1984b) developed procedures to analyze all glutenin subunits by RP-HPLC. Isolated glutenin or glutenin resulting from sequential extraction of single kernels is reduced with 2-mercaptoethanol and then alkylated with 4-vinyl pyridine. High-resolution separations are obtained for all glutenin subunits (Fig. 13). Glutenin's major high-MW group I subunits, closely associated with quality, elute early in the chromatogram, as determined through analysis of

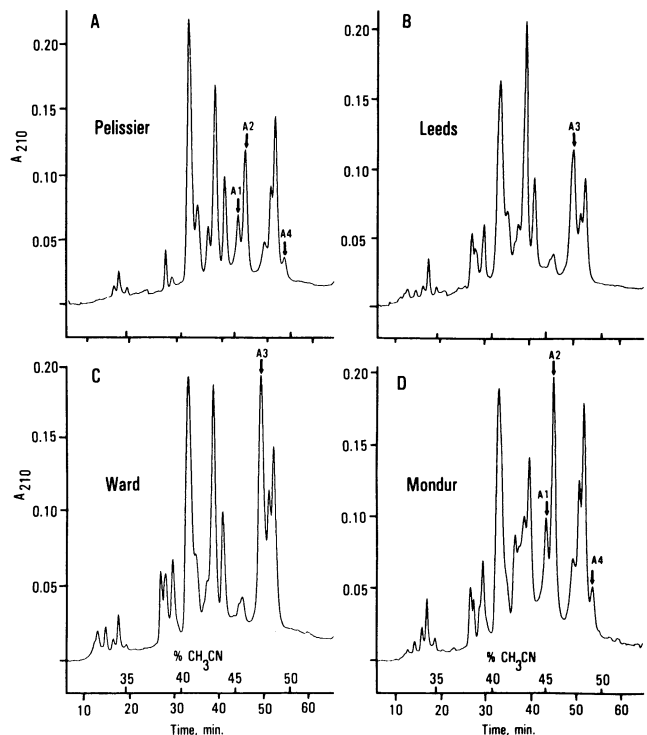


Fig. 10. RP-HPLC separation of durum wheat gliadins on SynChropak RP-P (250 × 4.1 mm) from single kernels of the varieties (A) Pelissier, (B) Leeds, (C) Ward, and (D) Mondur. Samples were eluted with a linear 20–55% B gradient during 55 min at 1 ml/min. Other conditions as in Figure 1. From Burnouf and Bietz 1984a.

wheat aneuploid lines; thus, RP-HPLC has potential for predicting breadmaking quality. In addition, qualitative and quantitative differences occur between varieties, suggesting that glutenin analysis may help differentiate varieties having similar gliadins.

RP-HPLC of Proteins from Other Cereals

RP-HPLC is valuable for analysis of proteins from all cereals, not only wheat. An example for the corn prolamin fraction, zein, is shown in Figure 14.

Wall et al (1984) showed that two-dimensional electrophoresis of zeins can differentiate corn inbreds and demonstrate genetic and evolutionary relationships. RP-HPLC can serve a similar role. Most corn inbreds, such as N28 and Mp490 shown here, can be identified by characteristic chromatographic patterns. The hybrid resulting from these two inbreds (Fig. 14C) closely resembles both parental lines and shows the predominant influence of the female parent. A "synthetic" chromatogram obtained by adding the two inbred patterns (Fig. 14D) closely resembles the actual hybrid (Fig. 14C). The same computer program can also subtract either inbred from the hybrid, leaving a chromatogram characteristic of the other parent. The ability to resolve a hybrid into parental lines, and to identify such lines, may be useful for registration and certification. It should also be possible to identify F₂ field-grown samples, just as it may be possible to identify varieties and mixtures of other cereals.

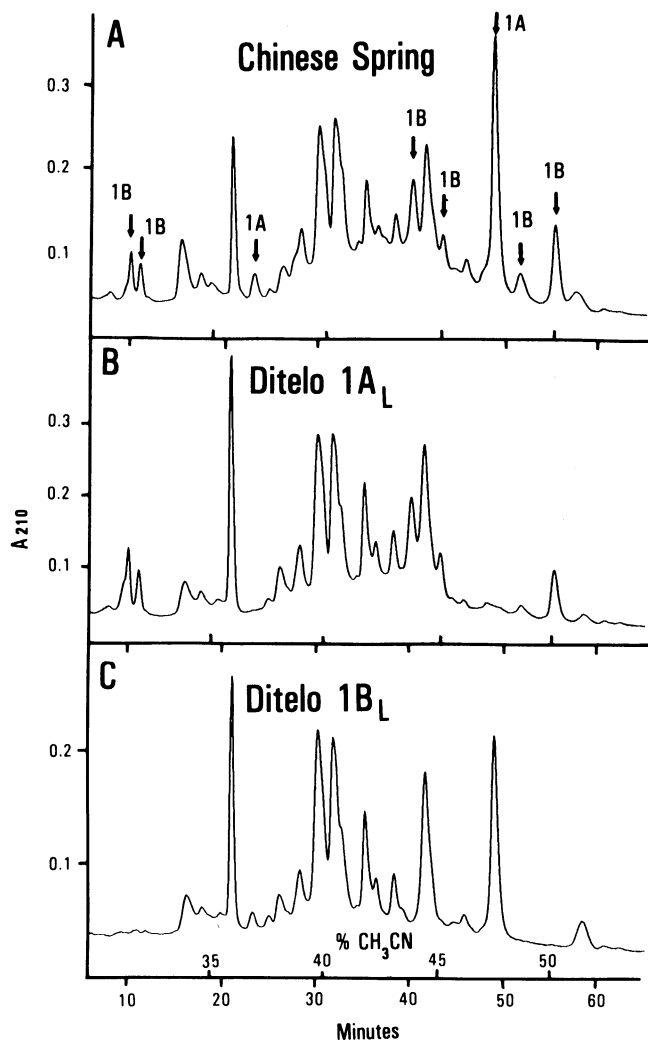


Fig. 11. RP-HPLC analysis of gliadins extracted from (A) Chinese Spring, (B) Chinese Spring ditelo 1A_L, and (C) Chinese Spring ditelo 1B_L. Arrows and chromosome designations in (A) indicate peaks absent in corresponding ditelo lines. Conditions as in Figure 1. From Bietz et al 1984b.

Other investigators also are finding RP-HPLC to be applicable to proteins from additional cereals. Summers et al (1983), as well as L. V. S. Sastry in conjunction with J. A. Bietz, J. S. Wall, and J. W. Paulis (*unpublished data*), analyzed sorghum kafirins by RP-HPLC and used the method for protein isolation and genetic comparisons. Similarly, Marchylo and Kruger (1984) reported that RP-HPLC can differentiate barley cultivars by hordein analysis. It is becoming apparent that RP-HPLC can isolate and analyze proteins from all cereal grains.

SE-HPLC OF CEREAL PROTEINS

Size-exclusion chromatography has been one of the most useful techniques for analyzing cereal proteins. Separations occur on the basis of molecular size: if proteins are larger than support pores, they rapidly elute from the column. Smaller proteins are retarded by the support in inverse relation to their molecular size. Proteins are thus sorted by size, and accurate MW estimates can be obtained from elution times.

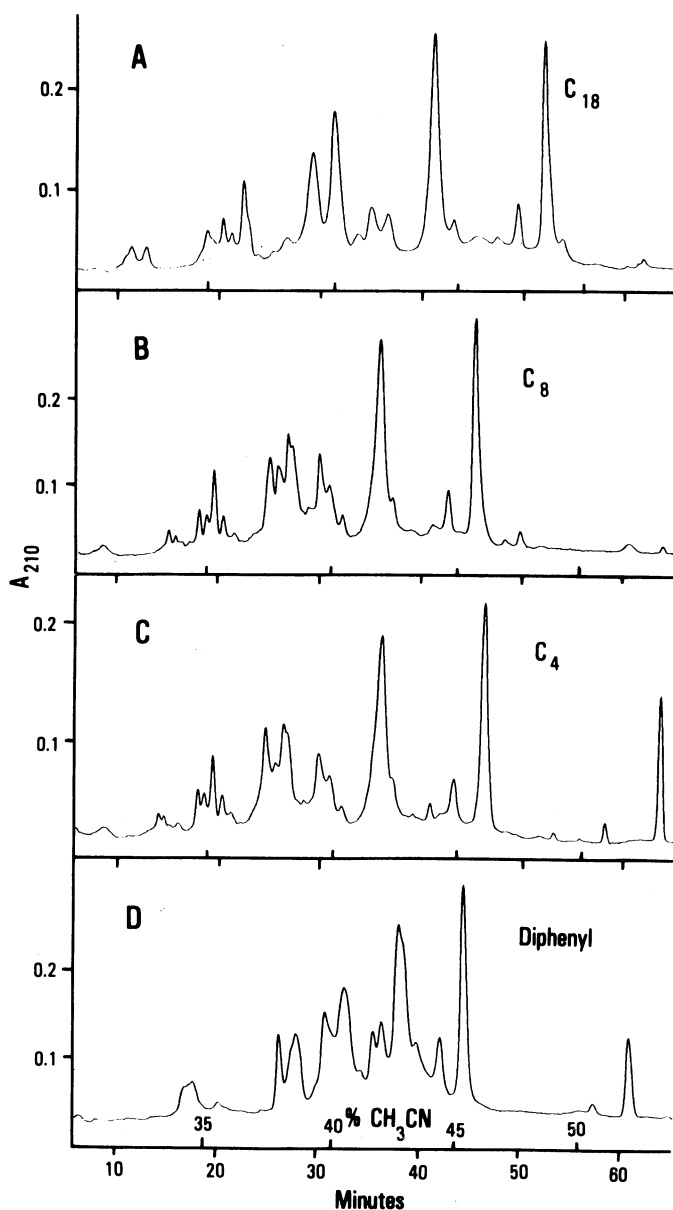


Fig. 12. Separations of gliadin proteins extracted with 70% ethanol from the wheat variety Era and analyzed on various RP-HPLC columns: (A) Bakerbond C₁₈, (B) SynChropak RP-P (C₈), (C) SynChropak RP-4 (C₄), and (D) Whatman Proteasil 300 diphenyl. Conditions as in Figure 1.

Conventional size-exclusion chromatography has serious drawbacks, however. It is slow, and column beds frequently are unstable. Results may be difficult to reproduce and quantitate. Thus, stable, uniform, and reproducible SE-HPLC columns seem to offer significant advantages for cereal proteins. SE-HPLC separations are fast, allowing larger numbers of samples to be analyzed. For these reasons, SE-HPLC has been used extensively to analyze cereal proteins (Bietz 1984a); this section reviews the procedures and presents typical results.

SE-HPLC supports vary in porosity, just as do conventional gel filtration materials. We have used three columns, TSK-4000SW, TSK-3000SW, and TSK-2000SW, prepared by Toyo-Soda. These columns have pore sizes of approximately 450, 240, and 130 Å, respectively, and are thus useful for high-, intermediate-, and low-MW proteins. Columns (500 × 7.5 mm i.d.) have exclusion volumes of 7–8 ml and total column volumes of approximately 22 ml; a guard column (TSK-3000SW, 100 × 7.5 mm i.d.) is also used. The solvent most used for SE-HPLC of cereal proteins is 0.1 M sodium phosphate, pH 7.0, containing 0.1% SDS. SDS binds to and solubilizes proteins, so that sizes of resulting protein-SDS complexes are related to protein MW. In the presence of SDS, TSK-4000SW separates proteins in the MW range 10,000–1,000,000, whereas TSK-3000SW separates proteins from approximately 10,000–200,000 MW; TSK-2000SW is used primarily for low-MW proteins and peptides. Flow rates are usually 1.0 ml/min (200–300 psi), and analyses are performed at room temperature. Samples of 10–50 μl, containing 0.5–3.0 mg/ml (1–10 μg) protein, are usually analyzed.

Before analysis, samples are complexed to SDS for 1 hr at 50–60°C in 0.1 M sodium phosphate, pH 7.0, containing 2.0% SDS, and centrifuged; reducing agent (2-mercaptoethanol) can be added to cleave disulfide bonds. Proteins can also be extracted directly from ground single wheat kernels to obtain either individual solubility classes or "total protein" extracts.

To determine MW by SE-HPLC, it is necessary to calibrate the column using known protein standards. When log MW is then plotted against elution time, a straight line is obtained, the equation for which can be used to estimate MW of unknown proteins. Standards are run daily and used to update computer programs that indicate elution times, areas, percentages, and apparent MW for each peak.

Analysis of Gliadins

Figure 15 presents SE-HPLC results for whole gliadin and for a purified gliadin fraction. Whole gliadin (Fig. 15A) has a major peak

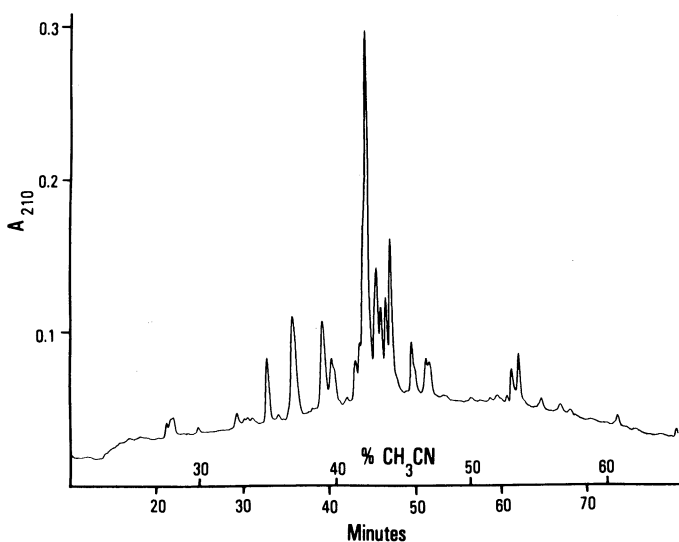


Fig. 13. RP-HPLC separation of glutenin subunits of the wheat variety Centurk. Glutenin was obtained via sequential extraction of a single kernel, reduced, alkylated with 4-vinylpyridine, and analyzed on a Bakerbond C₈ column using conditions as in Figure 1. From Burnouf et al 1983a.

(α , β , and γ gliadins) corresponding to MW 28,000, and smaller peaks and shoulders corresponding to MW 11,000, 41,000, 63,000 (ω gliadins), and 105,000 (high-MW gliadin). Resolution is similar to that of conventional gel filtration, and calculated MW agree with those from other techniques (Bietz and Wall 1972, 1980). Similarly, γ -gliadin (Fig. 15B) isolated by SEC chromatography has a nearly homogeneous MW of 43,000, agreeing with MW estimates from amino acid analysis and SDS-PAGE.

SE-HPLC of High-MW Gliadin

SE-HPLC can also compare proteins in their native and reduced states. High-MW gliadin is a heterogeneous series of oligomers intermediate in MW between gliadin and glutenin; its average MW is approximately 125,000. SE-HPLC (Fig. 16) gives results comparable to previous studies. Native high-MW gliadin (Fig. 16A) contains some proteins excluded from TSK-4000SW (apparent MW greater than 800,000), a distribution of proteins having MW in excess of 142,000, and small amounts of low-MW species. After reduction of disulfide bonds (Fig. 16B), the major peak corresponds to MW 41,000 (which is characteristic of ethanol-soluble reduced glutenin subunits), whereas other species similar in

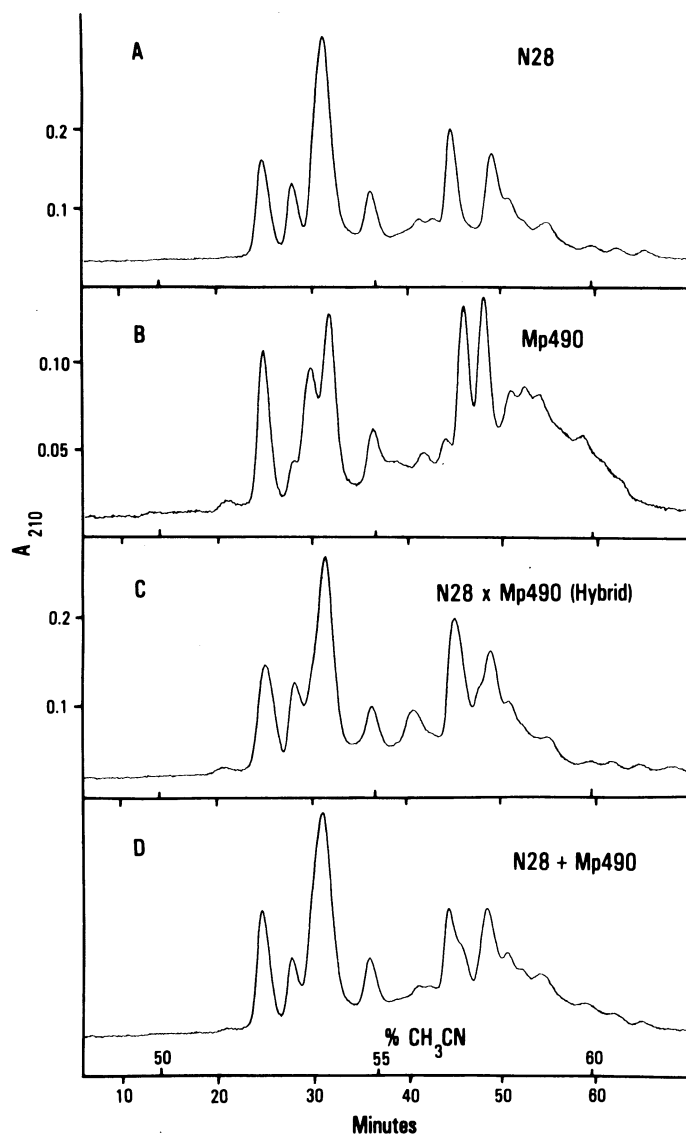


Fig. 14. RP-HPLC separation of 70% ethanol-soluble zein proteins from corn inbreds and hybrids. (A) N28; (B) Mp490; (C) N28 × Mp490 (hybrid); and (D) synthesized chromatogram of expected hybrid obtained through addition (in 2:1 ratio) of chromatograms (A) and (B). The chromatograms were developed with a linear gradient from 50 to 70% B during 60 min, and monitored at A₂₁₀ = 0.2 a.u.f.s.; other conditions as in Figure 1.

MW to reduced glutenin subunits are present in lesser amounts. Thus, SE-HPLC rapidly confirmed that high-MW gliadin is composed primarily of low-MW glutenin subunits joined through disulfide bonds.

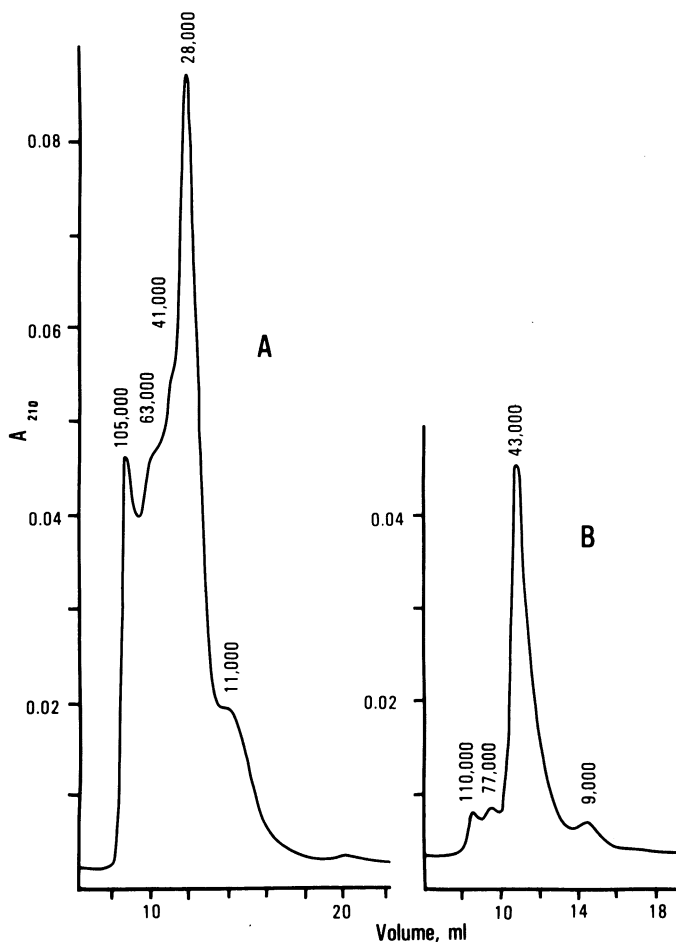


Fig. 15. Comparison of crude and purified wheat gliadins by SE-HPLC on TSK-3000SW: (A) whole native Ponca gliadin, (B) reduced γ -3 gliadin. Proteins were complexed to SDS and eluted from the column with 0.1 M sodium phosphate, pH 7.0, containing 0.1% SDS at 1.0 ml/min. Estimated MW are indicated for major chromatographic peaks. From Bietz 1984a.

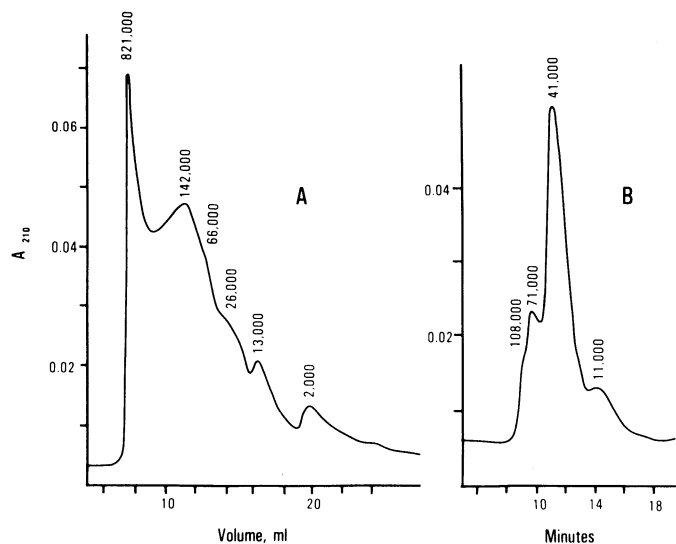


Fig. 16. Comparison of (A) native and (B) reduced high-MW Ponca wheat gliadin proteins by SE-HPLC on TSK-4000SW. Conditions as in Figure 15. From Bietz 1984a.

SE-HPLC Analysis of Glutenin Subunits

Previous studies have shown that glutenin consists of subunits having MW of approximately 100,000, 80,000, and 40,000; similar results can readily be obtained by SE-HPLC (Fig. 17A). SE-HPLC can also characterize and compare isolated subunit fractions. For example, glutenin subunits can be fractionated on the basis of their solubility in aqueous 70% ethanol (Bietz and Wall 1973). SE-HPLC (Fig. 17B, C) reveals that ethanol-soluble subunits are primarily of low MW, whereas the ethanol-insoluble fraction contains primarily high-MW subunits.

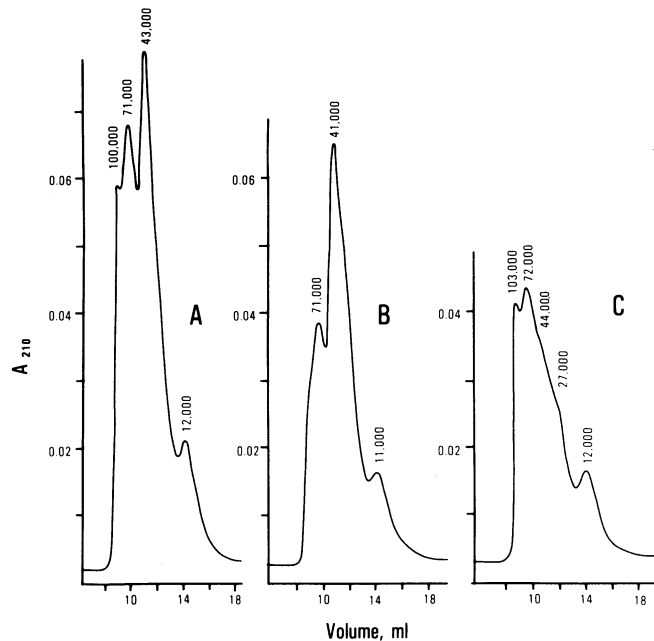


Fig. 17. Comparison of (A) reduced and aminoethylated glutenin subunits, (B) ethanol-soluble glutenin subunits, and (C) ethanol-insoluble glutenin subunits from Ponca wheat by SE-HPLC on TSK-3000SW. Conditions as in Figure 15. From Bietz 1984a.

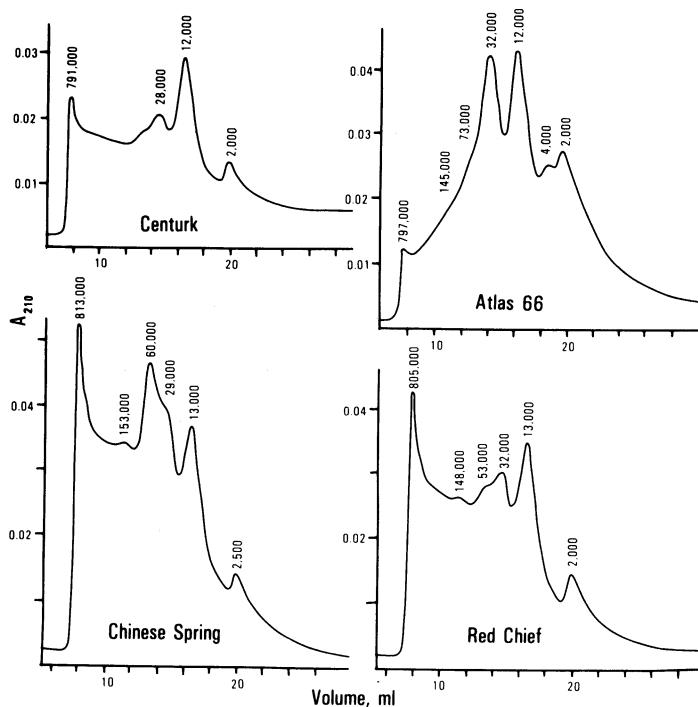


Fig. 18. Comparison of SDS-soluble native glutenin proteins extracted from single kernels of the wheat varieties Centurk, Atlas 66, Chinese Spring, and Red Chief, by SE-HPLC on TSK-4000SW. Other conditions as in Figure 15. From Bietz 1984a.

SE-HPLC of Native Glutenin

Because the MW distribution of native glutenin is closely associated with breadmaking quality, it would be desirable to characterize native glutenin by SE-HPLC. However, the MW of glutenin ranges up to 20,000,000, which is beyond the range of current columns. Nevertheless, SE-HPLC can give potentially important information about the structures of lower MW native glutenin molecules.

Following extraction of nonglutenin proteins, a portion of native glutenin was solubilized with SDS. Extracts from different varieties differed markedly in MW distribution (Fig. 18). For example, the extract from Atlas 66, a high-protein wheat, contained little high-MW protein but much low-MW protein, which seems to be noncovalently incorporated into glutenin. Similar extracts of Chinese Spring and Red Chief, both poor varieties, contained much high-MW protein. The extract from Centurk, a good bread wheat, has a more balanced ratio of high- and low-MW proteins. These results suggested a correlation between MW distribution of these extracted glutenins and quality, which was subsequently confirmed in flour samples differing in dough-mixing characteristics. Similarly, Orth and Bushuk (1972) found that wheat quality is inversely related to the amount of glutenin easily soluble in acetic acid. Thus, these results, although preliminary, suggest that rapid tests combining protein extraction and SE-HPLC should be able to predict bread wheat quality.

These examples have shown that SE-HPLC is valuable for determining cereal protein MW, for estimating purity, for comparing proteins, and for demonstrating disulfide or noncovalent bonds. Others are also using SE-HPLC for cereal protein separations: for example, Kruger (1984) analyzed the MW distribution of barley proteins by such a method. SE-HPLC may relate protein MW with flour quality and can differentiate varieties through analysis of proteins from single kernels, suggesting a role for SE-HPLC in breeding and genetic studies. Resolution of SE-HPLC seems no better than that of conventional gel filtration, but it is fast, sensitive, reproducible, and easy to quantitate. SE-HPLC promises to be valuable for cereal protein analysis from breeding to final utilization.

IE-HPLC OF CEREAL PROTEINS

Ion-exchange chromatography, primarily on cellulosic supports, has been one of the best techniques for fractionating cereal proteins (Huebner and Rothfus 1968, Charbonnier 1974). Thus, IE-HPLC columns, having functionalities similar to standard columns but using silica supports rather than cellulose, dextrans, or resins, promise to be valuable for cereal protein isolation and characterization. IE-HPLC columns should be more stable than traditional supports, as well as more uniform chemically, eliminating nonionic adsorption; resolution should increase due to the use of small, uniform, porous support particles.

To date, very few applications of IE-HPLC to cereal protein separations have been examined. One example is shown in Figure 19. A sample of Centurk wheat flour was extracted with 0.1M NaCl; an aliquot was injected onto a CM-300 (SynChrom) weak cation exchange column and eluted at pH 7.0 with a sodium phosphate buffer gradient (0.02–0.31M). These results demonstrate that numerous proteins, in this case endosperm albumins and globulins, can be separated by IE-HPLC, and suggest that many further applications for cereal proteins will be found. For example, Batey (1984) demonstrated wheat varietal identification by a "fast protein liquid chromatography" anion-exchange technique.

SUMMARY, CONCLUSIONS, AND FUTURE

These examples, and the studies from which they are taken, clearly show that RP-, SE-, and IE-HPLC are well suited for analysis of cereal proteins. The methods are fast, sensitive, and give high-resolution separations. Columns are stable and give highly reproducible separations. Analyses may be easily automated and results accurately quantitated.

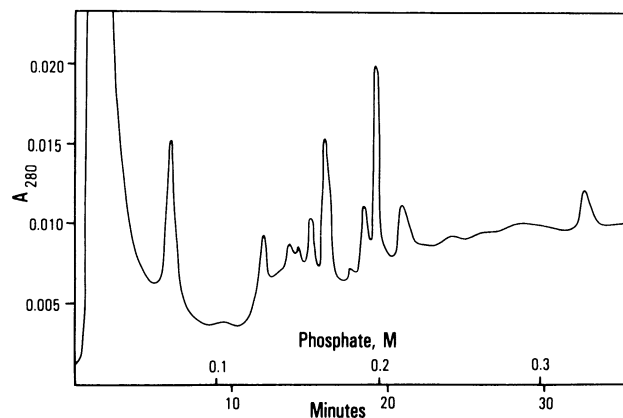


Fig. 19. Cation-exchange HPLC on SynChrom CM-300 of albumin and globulin proteins extracted from Centurk wheat flour with 0.1M NaCl.

To date, HPLC has been found useful for isolation, characterization, and comparison of cereal proteins. Results may be used for varietal identification and may demonstrate varietal purity. In breeding and genetic studies, HPLC may permit selection for specific characteristics through analysis for known marker proteins, as well as selection for atypical genotypes. Computer techniques should permit identification of parental lines of hybrids and analysis of components in mixtures. Both RP- and SE-HPLC can measure cereal quality characteristics; as additional proteins that relate to desirable characteristics are detected, it should be possible to devise additional rapid methods to predict quality.

It is inevitable that HPLC will become widely used for analysis of cereal proteins. Current separations are very good, but further improvements in column technology continue to improve resolution and decrease separation times. In the future, we should be able to use HPLC to isolate, characterize, and compare cereal proteins and to use the resulting information to predict, measure, improve, and assure good quality of all cereals and cereal-based products.

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