Improvements in Wheat Protein Analysis and Quality Prediction by Reversed-Phase High-Performance Liquid Chromatography[1]

F. R. HUEBNER and J. A. BIETZ[2]

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ABSTRACT

Specific problems in wheat gluten protein analysis by reversed-phase high-performance liquid chromatography (RP-HPLC) and in evaluating data for predicting quality were found. Procedures were developed to improve the method's reliability and reproducibility. To obtain accurate integration, a flat baseline is desirable; this was achieved by reducing (15–18%) the concentration of trifluroacetic acid in acetonitrile. The similarity of gliadins extracted with 50 or 70% ethanol or after pre-extraction with 0.1*M* NaCl was demonstrated; adjustment of extractant hydrophobicity prevented early elution of gliadins during preparative RP-HPLC. Long-term storage of gliadins at various temperatures was studied; −70°C is recommended. Various dissociating solvents for extraction, reduction, and alkylation of glutenin were compared, and the need for acidification following alkylation for long-term storage was demonstrated. We also demonstrated that computer analyses of chromatographic data can identify proteins correlated with quality.

Wheat flour has been tested for many years for its breadmaking quality (Bloksma 1971); nevertheless, more accurate and sensitive analytical methods are always welcome. For example, the development of a new mixograph reduced flour requirement to 10 g (Finney and Shogren 1972), and a sodium dodecyl sulfate (SDS) sedimentation method, which gave results somewhat similar to the farinograph, used as little as 5 g of flour (Moonen et al 1982, Preston et al 1982). Also, gel electrophoresis of samples as small as single kernels now permits estimation of wheat baking quality based on presence or absence of specific glutenin subunits (Payne et al 1979, Branlard and Rousset 1980; Wrigley et al 1981.

Reversed-phase high-performance liquid chromatography (RP-HPLC) can also examine specific wheat proteins and estimate quality from 50 mg of flour or less (Burnouf and Bietz 1984a,b; Huebner and Bietz 1985). While this method is fast, quantitatively accurate, and easy, some problems still need to be solved. This paper identifies some of these problems, and discusses how they can be avoided.

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[2]Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604.

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MATERIALS AND METHODS

Materials

Flour samples of hard red spring wheat (milled on a Buhler continuous experimental mill) were obtained from the USDA Spring and Durum Wheat Quality Lab, Fargo, ND, and from the Department of Cereal Science and Food Technology, North Dakota State University, Fargo. Wheats were grown in test plots in North Dakota, Arizona, and California in 1983 or 1984. Data were supplied for protein content, mixing time, loaf volume, milling characteristics, absorption, crumb color and grain, dough character, and bake score (Nolte et al 1985).

Solvents used were HPLC grade, and other chemicals were reagent grade or better, as described previously (Bietz 1983). For some samples, a micropartition centrifugation device (Amicon, Danvers, MA), with membranes of 10,000 or 30,000 mol wt cutoff, was used to quickly remove salts and reagents from samples.

Sample Preparation

Flour samples (50 mg) were extracted in 10-ml capped polypropylene centrifuge tubes for 20–60 min with continuous agitation on a vortex evaporator (Buchler Instruments, Fort Lee, NJ). Extractions were at room temperature unless stated otherwise. After extraction, samples were centrifuged for 15 min at 15,000–20,000 rpm (30,000 × *g*) in a Beckman L8-70M centrifuge (Beckman Instruments, Fullerton, CA) with a type 40 rotor. Glutenin was prepared (Huebner and Bietz 1985) by sequentially extracting albumins, globulins, and gliadins first with 0.4*M* NaCl and then with 70% ethanol. The residue was suspended in 70% ethanol containing 0.1*M* acetic acid for 30 min and agitated. The suspension was then adjusted to pH 6.5 to precipitate glutenin and remove any additional associated nonglutenin proteins. Remaining glutenin was reduced with 0.1% (w/v) dithiothreitol (DTT) for 1.5 hr and alkylated for 1 hr with 4-vinylpyridine at room temperature. For some experiments (as described below), the

alkylated glutenin subunit/starch mixture was acidified (Burnouf and Bietz 1984b) for storage.

RP-HPLC

RP-HPLC was performed with a Spectra-Physics (San Jose, CA) SP8700 solvent delivery system and SP8780XR autosampler, a Schoeffel SF770 spectroflow monitor (Kratos, Ramsey, NJ), and a 250 × 4.1 mm SynChropak RP-P (C_{18}) column (SynChrom Inc., Linden, IN). Column temperature was kept constant with a model CH-20-C column heater (Scientific Systems, State College, PA). A 22 × 3.5 mm guard column (SynChropak RSC) and 0.5-μm in-line prefilter (A-103, Upchurch Scientific, Oak Harbor, WA) were also used. Eluted components were detected at 210 nm at 0.1 absorbance units full scale (AUFS)/10 mV and recorded on an OmniScribe recorder (Houston Instruments, Austin, TX). Data were simultaneously stored in a ModComp computer system (model Classic 7870; Ft. Lauderdale, FL) for subsequent integration and/or replotting.

Gradients were generated from solvents A (water containing 0.06% trifluoroacetic acid [TFA]) and B (acetonitrile [ACN] containing 0.05% TFA). Before use, solvents were filtered through a 0.45-μm (HVLP) Millipore filter, deaerated under vacuum, and sparged with a rapid flow of helium for 10–15 min. During use, slow sparging with helium was continued to provide slight back pressure in solvents stored in capped bottles with small diameter (100 cm long) exit tubes (Huebner and Bietz 1985).

Samples of 10–20 μl were analyzed at 1.0 or 1.1 ml/min at 70° C. Gradients were linear for most long runs, but in some cases steeper initial gradients were used to provide more rapid elution of the early eluting components (Huebner and Bietz 1985).

Computation

Chromatographic data were integrated with a computer program TIMCPC (developed by R. Butterfield, USDA/ARS Northern Regional Research Center, Peoria, IL). Comparisons were facilitated by the ability to specify exact elution time ranges, resulting in comparable integration of data for each sample. Linear regression analyses were performed and plotted by the program LINREG (Huebner and Bietz 1985).

RESULTS

Effects of TFA Concentration

For RP-HPLC of proteins and peptides, gradients of water and ACN containing 0.1% TFA are generally used. Absorbance at 210 nm varies with solvent purity, and ACN generally absorbs more than water. Gradient baselines (Fig. 1A) may therefore slope upward, particularly at high sensitivities (< 0.1 AUFS), interferring with integration.

To alleviate this problem, a stored baseline may be subtracted from chromatograms by computer. Others equalize absorbance of RP-HPLC solvents by adding butane-2-one (Sjodahl 1979). Because TFA has high absorbance at 210 nm, small differences in TFA concentrations can also compensate for different absorbances of water and ACN. For example, 0.06% TFA in water and 0.05% TFA in ACN (Fig. 1A) generally gives a flat baseline; slight modification may be necessary for different lots of ACN. Lower TFA concentrations also reduce the possibility of deamidation (Huebner and Bietz 1984) of glutamine, which makes up approximately 33% of the amino acids in gliadin. Figure 1B compares gliadin chromatograms obtained with constant TFA (0.1%) or with 0.06% TFA in water and 0.05% TFA in ACN; an improved baseline is achieved by varying TFA concentration.

Changing the TFA concentration also alters gliadin elution times (Fig. 1B and C), so comparable TFA concentrations must be used to compare samples. If 0.1% rather than 0.05% TFA is used, flat baselines result using about 0.1% and 0.085% TFA in water and ACN, respectively.

Extraction Solvents

In previous studies (Bietz 1983, Bietz et al 1984, Burnouf and Bietz 1984a), gliadin was extracted with 70% ethanol and 10–30 μl aliquots were applied to the RP-HPLC column. If, however, the ultraviolet detector does not have adequate sensitivity, or if 280 nm is used instead of 210 nm as in preparative RP-HPLC (Huebner and Bietz 1984), larger samples must be applied. Under these conditions, the high ethanol content of samples causes protein to elute at the void volume, because ACN and ethanol have similar solvent strengths (Mahoney and Hermodson 1980), and initial gradient conditions for gliadins are generally above 20% ACN (Fig. 1). We have now found that gliadins may be extracted with 50% as well as 70% ethanol (Fig. 2). Samples were not as stable in

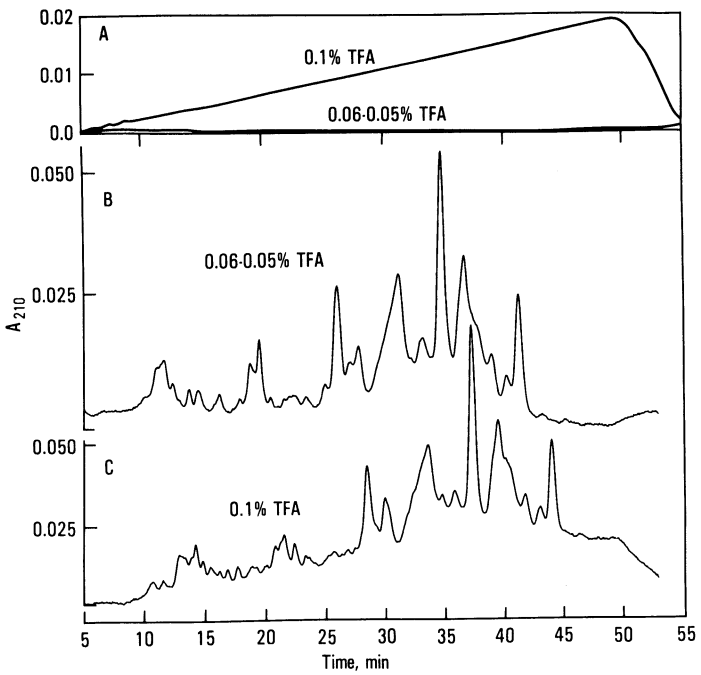

Fig. 1. Effect of trifluoroacetic acid (TFA) concentration on baseline slope and gliadin separations. All chromatograms were obtained with solvents H_2O and acetonitrile (ACN) with indicated concentrations of TFA. (**A**) Blank runs at constant (0.1%) TFA concentration and with 0.06% TFA in H_2O and 0.05% TFA in ACN. (**B**) 70% ethanol extract from hard red spring wheat flour (variety Iuanillo) analyzed with 0.06% TFA in H_2O and 0.05% TFA in ACN. (**C**) 70% ethanol extract from Iuanillo flour analyzed at constant (0.1%) TFA concentration. Gradient: 21–45% ACN during 40 min, with a 5-min final hold. Flow rate, 1.0 ml/min.

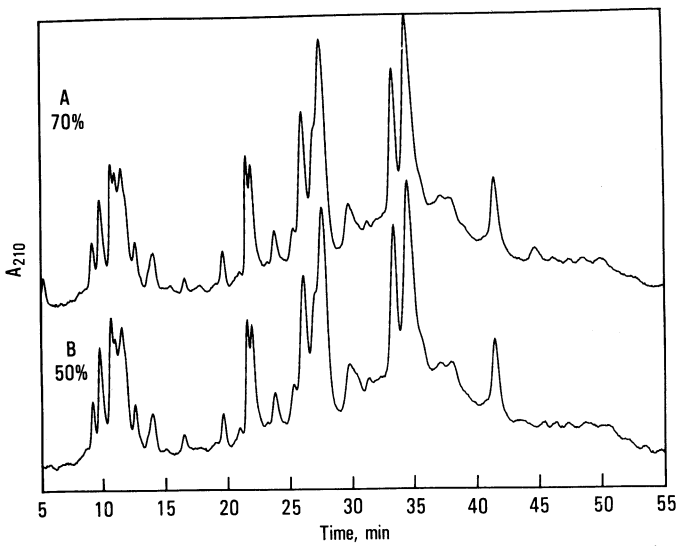

Fig. 2. Reversed-phase high-performance liquid chromatography of gliadin (variety Oslo) extracted with 70% (**A**) and 50% (**B**) aqueous ethanol. The gradient was from 26 to 46% acetonitrile (ACN) during 40 min with a 5-min final hold, with 0.06% and 0.05% trifluoroacetic acid in H_2O and ACN, respectively. Other conditions were as in Fig. 1.

50% ethanol; however, coextracted proteases may remain active in 50% ethanol, or gliadins may associate and precipitate after storage. When applying large samples, using 50% instead of 70% ethanol decreases the tendency of proteins to elute prior to initiation of a gradient; solvents of still lower hydrophobicity may be desirable (Huebner and Bietz 1984). Columns may also be equilibrated to a lower (e.g., 10%) acetonitrile concentration, and after sample is applied be re-equilibrated to starting gradient conditions or a nonlinear gradient used. For small samples (< 50 μl) in 70% ethanol, no gliadins elute at the void volume for a 25-cm long column.

We also compared 70% ethanol extraction with extraction following 0.1*M* NaCl, and with 0.1*M* NaCl extraction alone. Extraction with dilute NaCl solubilizes proteins having gel electrophoresis mobilities (in aluminum lactate) similar to gliadin, as well as faster-migrating albumins and globulins (data not shown). RP-HPLC (Fig. 3) reveals that 0.1*M* NaCl also extracts components with hydrophobicities similar to gliadin, as noted previously by Bietz (1983). Since, however, albumins and globulins are minor components of direct 70% ethanol extracts, RP-HPLC results for 70% ethanol extracts with or without previous NaCl extraction are similar, except for minor quantitative variation among early peaks. Similar results were noted by Bietz et al (1984). Gliadins are also somewhat soluble in water (Bernardin and Kasarda 1973), and may be slightly soluble in 0.1*M* NaCl. Thus, extraction methods can affect results, and should be standardized to compare gliadins from different varieties.

Sample Storage

In other studies (Bietz et al 1984, Huebner and Bietz 1986), gliadins were stable in 70% ethanol when stored at room temperature. Because noticeable changes occasionally occurred upon prolonged storage, sample storage under various conditions was investigated. After 64 days at −15° C, there were no major differences between chromatograms of the stored and fresh extracts (Fig. 4); minor differences were noted only at 23 min and in the ratio of peaks at 26–28 min. Comparison of a sample stored at −70° C to a fresh extract revealed a minor difference only at 33–34 min. However, samples stored at room temperature for 30 days had a new peak at about 5 min; this peak increased with time, becoming quite large at 64 days (Fig. 4D). The sample stored for 64 days at room temperature also differed from the fresh extract, especially between 20 and 25 min and after 30 min.

Storage stability of glutenin subunits was studied by Burnouf and Bietz by RP-HPLC (1984b). Acidified reduced and alkylated glutenin subunits were stable for at least one month at room temperature, as evidenced by reproducible separations. If samples are not sufficiently acidified and alkylating reagents are left in sample solution, however, results may vary with storage time (Fig. 5). Even though stored at −70° C, changes took place quite rapidly, particularly among early eluting high-molecular-weight subunits (Burnouf and Bietz 1985). The first major peak nearly disappeared in 24 days, and the ratio of the two tallest peaks changed. Also, lower molecular weight, more hydrophobic subunits (after 15 min) eluted earlier and were less resolved in older samples. Exact reasons for these changes are unknown; possibly proteins react with remaining alkylating reagents when samples are insufficiently acidified.

Dialysis or membrane filtration to remove reagents from proteins did not give reproducible results for stored samples, suggesting that the conditions of Burnouf and Bietz (1984b) may be optimal for stabilizing reduced and pyridylethylated glutenin subunits. Considerable care is necessary to ensure minimal

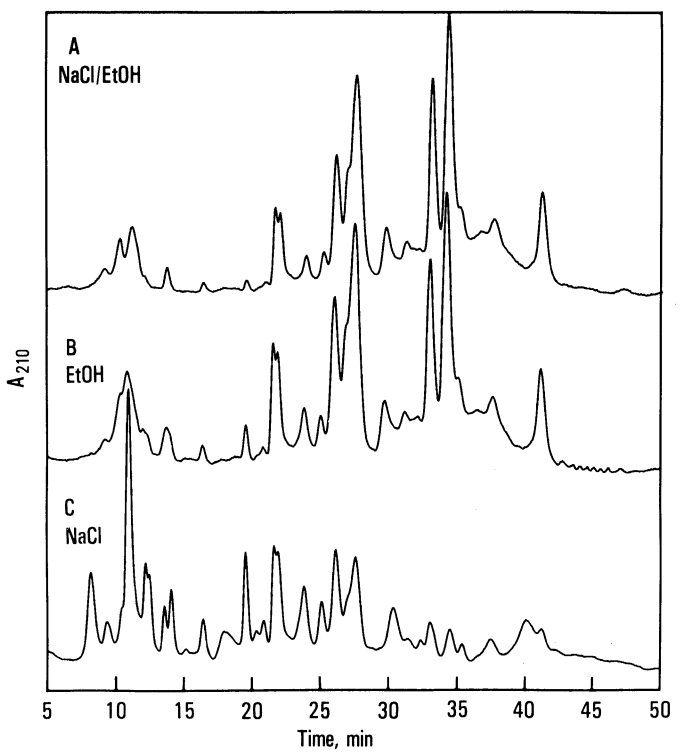

Fig. 3. Reversed-phase high-performance liquid chromatography of proteins extracted from the wheat variety Oslo with (**A**) 0.1*M* NaCl followed by 70% ethanol, (**B**) 70% ethanol, and (**C**) 0.1*M* NaCl. Absorbance scales have been normalized to facilitate qualitative comparison of samples. Solvent and gradient were as in Fig. 2.

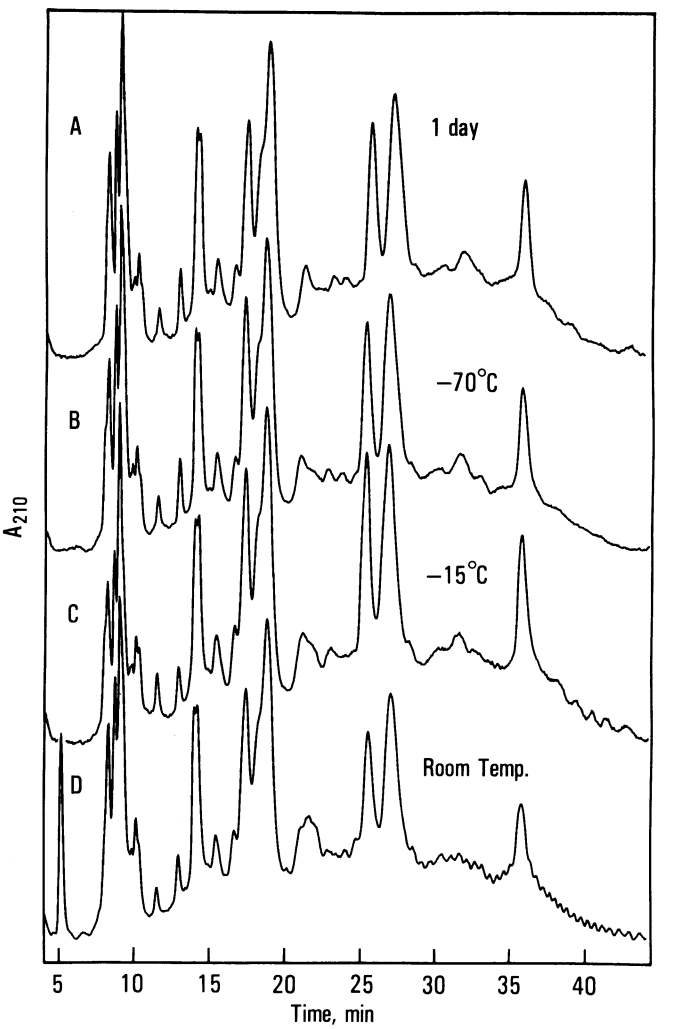

Fig. 4. Reversed-phase high-performance liquid chromatography of a 70% ethanol extract of wheat flour (variety Oslo) stored for (**A**) one day at room temperature, (**B**) 64 days at −70° C, (**C**) 64 days at −15° C, and (**D**) 64 days at room temperature. Chromatographic conditions were: flow rate, 1.1 ml/min; gradient: initial conditions, 24% acetonitrile (ACN); 0–1 min, 24–27% ACN; 1–3 min, 27–32% ACN; 3–6 min, 32–35% ACN; 6–28 min, 35–44% ACN; and 28–33 min, 44–46% ACN. Solvents contained 0.06 and 0.05% trifluoroacetic acid in H_2O and ACN, respectively.

degradation of pyridylethylated proteins by alkylating solvent; acidification to approximately pH 3.0 (Burnouf and Bietz 1984b) plus cold storage generally prevent such degradation.

Solvent for Glutenins

In an HPLC study of glutenin (Huebner and Bietz 1985), various solvents were tested for reduction, avoiding high urea and guanidine hydrochloride (GuHCl) concentrations. Figure 6 compares glutenin extracted with and reduced in the presence of 1.5% SDS to glutenin reduced, alkylated, and extracted in the presence of 4*M* GuHCl or urea under different temperatures.

Qualitative and quantititative differences occurred in ratios of high- to low-molecular-weight subunits (Burnouf and Bietz 1985, Huebner and Bietz 1985), particularly in the presence of SDS (Fig. 6A). SDS, although an effective extractant, does not dissolve all glutenin (Huebner and Wall 1980). Bietz (1983) noted excellent RP-HPLC resolution of gliadins in the presence of SDS, but elution times differed. Burnouf and Bietz (1984b) compared 2% SDS to 8*M* urea or 6*M* GuHCl to dissociate glutenin: resolution with SDS was inferior, and early (high-molecular-weight) peaks were absent. Our results reducing glutenin in the presence of SDS were somewhat inferior and not reproducible, suggesting considerable caution in using SDS in RP-HPLC analysis of glutenin. There is also some indication that SDS may be detrimental to a RP-HPLC column; however, we have no proof of this.

Glutenin RP-HPLC patterns also vary after reduction in the presence of low concentrations of urea (Fig. 6B) or GuHCl (Fig. 6C and D). It may be desirable to avoid high concentrations of urea or GuHCl to prevent starch gelatinization and minimize amounts of reagents injected with samples. Although 4*M* GuHCl may not fully disaggregate glutenin at room temperature (Fig. 6C), reduction at 50° C (Fig. 6D) produces additional peaks at 36–40 min and fewer between 16 and 28 min, indicating more complete reduction even though the starch may also gelatinize. Resolution of glutenin subunits reduced in 4*M* urea (Fig. 6B) also differs slightly from that of glutenin reduced in the presence of GuHCl.

We also found that glutenins that are soluble or insoluble in 3*M* GuHCl have similar ratios of high- to low-molecular-weight subunits (results not shown). In contrast, glutenins extracted with SDS or urea differed in ratios of high- to low-molecular-weight subunits (Huebner and Bietz 1985) from insoluble glutenins. Because native glutenins are not uniform in composition and differ subtly in their properties and contributions to breadmaking quality, our results demonstrate the necessity of carefully choosing conditions for glutenin solubilization, denaturation, and reduction to avoid incorrect interpretations. To solubilize, reduce, and analyze all glutenin species simultaneously, however, complete denaturation (i.e., in the presence of 8*M* urea or 6*M* GuHCl) is desirable (Burnouf and Bietz 1984b).

Use of Computers to Predict Quality

RP-HPLC generates a large amount of data for each sample, and many samples are easily analyzed. Computers are necessary to adequately store data, perform integrations and calculations, detect nonobvious relationships to quality, and determine correlation coefficients through linear regression analysis.

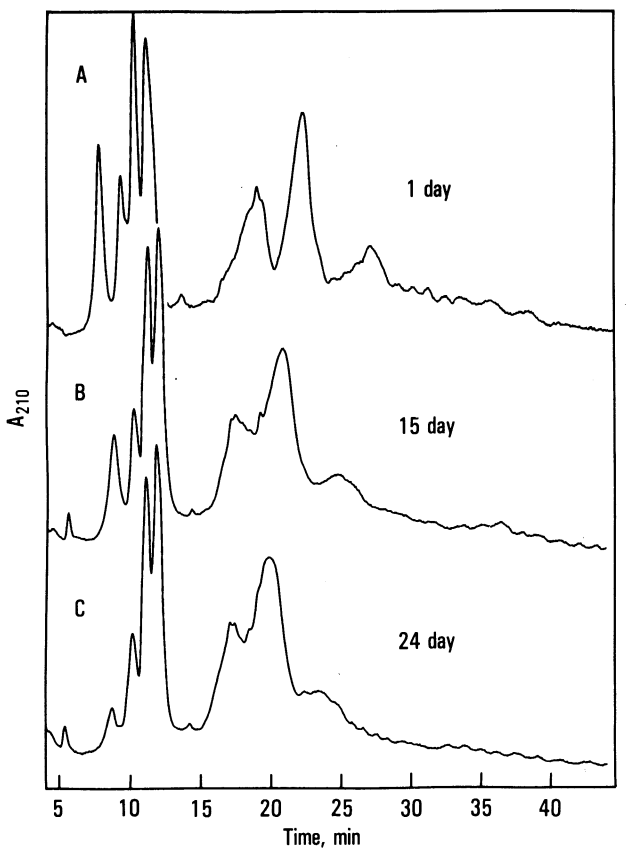

Fig. 5. Reversed-phase high-performance liquid chromatography of glutenin subunits. Glutenin (variety Coteau) was reduced with 0.1% DTT for 1.5 hr in 1 ml of 4*M* urea-0.05*M* sodium phosphate buffer, pH 7.6, alkylated with 4-vinyl pyridine for 1 hr at room temperature, acidified with 50 μl of 50% acetic acid (pH 4.0 ± 0.2), and stored at −70° C for one, 15, and 24 days. Gradient: 0–2 min, 25% acetonitrile (ACN); 2–4 min, 25–33% ACN; 4–6 min, 33–35% ACN; 6–29 min, 35-42% ACN; and 29–33 min, 42–44% ACN. Solvents and other chromatographic conditions were as in Fig. 2.

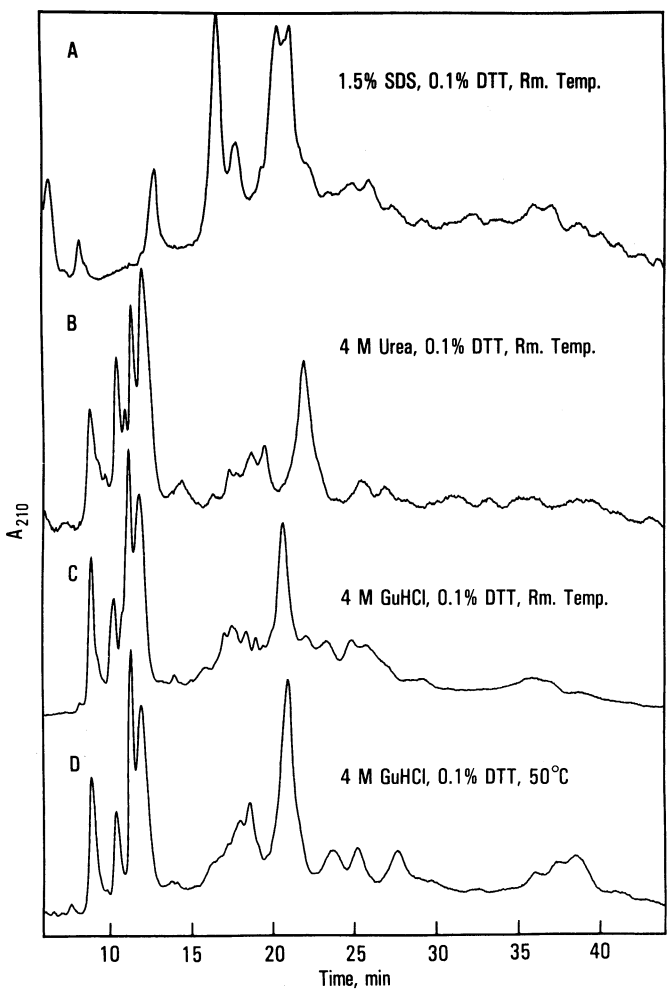

Fig. 6. Reversed-phase high-performance liquid chromatography of glutenin subunits (variety Coteau) solubilized by reduction under different dissociation conditions. (**A**) Reduction in 1.5% sodium dodecyl sulfate at room temperature; (**B**) reduction in 4*M* urea at room temperature; (**C**) reduction in 4*M* guanidine hydrochloride (GuHCl) at room temperature; (**D**) reduction in 4*M* GuHCl at 50° C. Other conditions were as in Fig. 5.

Figure 7 and Table I suggest how use of a computer can relate durum wheat quality (Burnouf and Bietz 1984a) to RP-HPLC data. Relative areas of three major gliadin peaks (A_1, A_2, and A_3 in Fig. 7) in six varieties were determined, and the areas were correlated with quality by linear regression analysis. Peak A_1 (not related to gluten strength) was poorly correlated with quality (r = 0.30), whereas peaks A_2 and A_3, which correspond to γ-gliadins "45" and "42" in the nomenclature of Damidaux et al (1978) correlate highly with quality (0.97 and −0.98, respectively). Analysis of all peaks in a series of RP-HPLC runs could be similarly automated, providing correlation coefficients for each component of a complex mixture.

Computer analysis may also correlate peak heights (i.e., detector voltage output) with quality reducing problems in integration when baseline resolution is not achieved or elution times vary slightly (Table I). Linear regression analysis of quality versus absorbance at times t_1, t_2, and t_3 (the maxima of peaks A_1, A_2, and A_3 in Fig. 7) gives results (r = 0.11, 0.97, and −0.96, respectively) equivalent to those obtained from integrated data. Plotting correlation coefficients as a function of time gives a visual representation of the entire chromatogram in which elution times associated with high positive or negative correlation coefficients identify components related to quality.

It is likely that this type of computer analysis will be able to identify unknown quality-related proteins in other wheat classes (such as hard red spring wheat gliadins correlated with quality [Huebner and Bietz, in press]) and cereal grains. Such proteins could then be isolated and characterized, leading to a better understanding of the relation of structure to functionality, and to rapid tests for quality.

TABLE I
Correlations Between Peak Areas or Heights and Quality

Wheat Variety	Quality[a]	Peak Area			Relative Peak Height[b]		
		A_1	A_2	A_3	t_1	t_2	t_3
Produra	0	16.3	3.0	16.1	12,080	1,239	11,064
Leeds	0	17.3	3.4	13.7	10,722	1,406	8,900
Calvin	0	14.5	1.9	16.5	9,210	890	12,455
Quilafen	1	16.0	10.3	7.1	11,556	8,012	3,496
Wakooma	1	19.3	9.8	5.6	11,961	8,100	3,165
Blondur	1	15.5	13.0	6.7	9,287	11,277	3,648
Correlation coefficient		+0.30	+0.97	−0.98	+0.11	+0.97	−0.96

[a] Arbitrarily defined as 0 = weak and 1 = strong.
[b] Relative voltage corrected to constant area/sample.

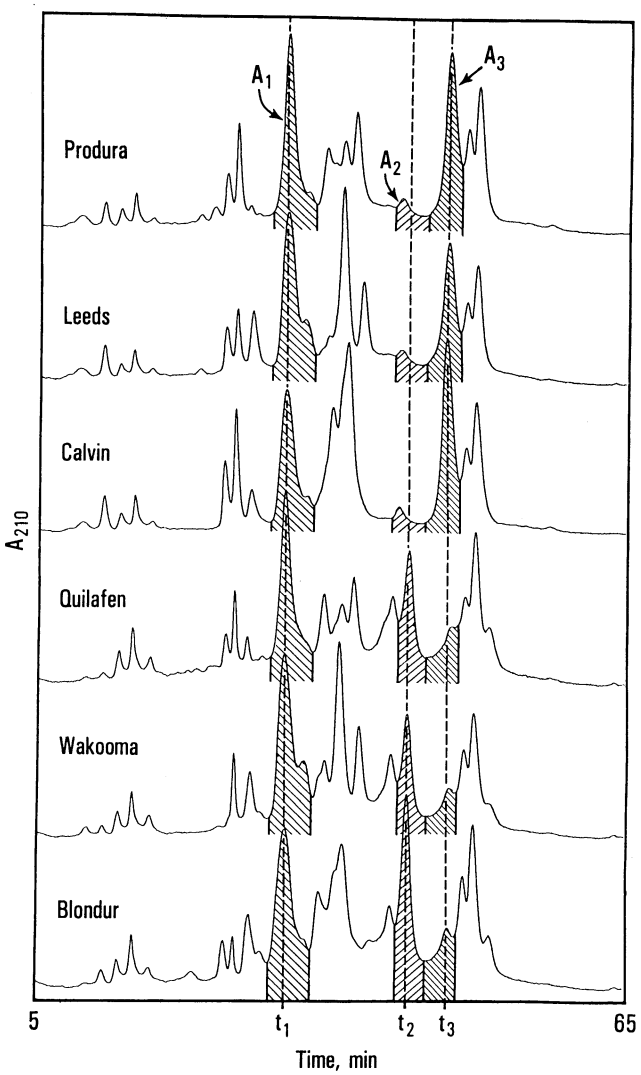

Fig. 7. Reversed-phase high-performance liquid chromatography of gliadins extracted with 70% ethanol from weak (Produra, Leeds, and Calvin) and strong (Quilafen, Wakooma, and Blondur) durum wheat varieties. A linear gradient from 28–51% acetonitrile (0.1% trifluoroacetic acid) during 55 min (total run time, 65 min) at 1.0 ml/min was used. A_1, A_2, and A_3 identify areas of peaks eluting at times t_1, t_2, and t_3, respectively. Column, SynChropak RP-P (C_{12}); temperature, 30° C.

DISCUSSION

RP-HPLC of cereal proteins has developed so rapidly that many techniques have not been optimized; therefore, we further examined sample preparation, stability, and analysis. Baseline shifts, a problem at high sensitivity, can be controlled by adjusting TFA concentrations. Similarly, 50% ethanol extracts gliadins equivalent to those solubilized with 70% ethanol, preventing premature elution when large volumes of samples are applied. For gliadin solubilization, direct extraction with 70% ethanol is generally preferable to use of preliminary extraction with NaCl solution.

Analysis of glutenin is more complex than that of gliadin. Derivitization reagents may remain reactive, decreasing sample stability; proper acidification and storage of samples largely alleviates this problem. Alkylation may be incomplete if denaturation conditions are inadequate. Amount and composition of glutenin solubilized may vary with extraction and denaturation conditions. Special precautions are thus necessary to prevent or minimize problems that have become apparent only because of the sensitivity and quantitative capabilities of RP-HPLC.

Computer analysis is necessary to fully use the large amount of data generated by RP-HPLC. Using the example of durum gluten strength, we demonstrated that the presence of amount of specific gliadins can be statistically correlated with measured quality characteristics. Similar analyses of other wheat proteins should indicate new relationships of protein type or amount to quality, further enhancing the value of RP-HPLC and its usefulness in optimizing wheat quality and utilization.

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