Wheat Varietal Identification and Genetic Analysis by Reversed-Phase High-Performance Liquid Chromatography[1]

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ABSTRACT

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Gliadins are a heterogeneous group of alcohol-soluble wheat endosperm storage proteins whose composition varies among wheat genotypes. Reversed-phase high-performance liquid chromatography (RP-HPLC) on large-pore columns gives high-resolution separations of gliadins from extracts of wheats, thereby permitting varietal identification and complementing other methods. Gliadins, extracted with 70% ethanol from single kernels or from bulk samples, are injected onto a reversed-phase (C_{18}) column having 300-Å pores. The proteins are eluted with a gradient of increasing acetonitrile concentration, and are detected at 210 nm. Resulting chromatograms reveal approximately 30–40 components. Examples are presented that demonstrate that significant qualitative and quantitative differences occur between nearly all hard red winter, hard red spring, soft red winter, white, and durum wheats examined; only varieties having nearly identical pedigrees may be impossible to differentiate. Analysis of aneuploids permits determination of the chromosomal locations of genes that code proteins eluting in each chromatographic peak, thus establishing markers for the chromosomes. Analysis of phenotypic variants from a sample also permits detection of different genotypes, biotypes, or other off-types. RP-HPLC is fast, sensitive, exhibits good run-to-run reproducibility, has high resolving power, and is easily automated; the ability to accurately quantitate data may facilitate computer-assisted chromatographic comparisons. RP-HPLC of gliadins can be a valuable tool for identification, selection, and comparison of genotypes in breeding and genetic studies.

The endosperm proteins of wheat are a mixture of unusual complexity and heterogeneity. Four distinct classes of proteins (albumin, globulin, gliadin, and glutenin) are present. Extensive heterogeneity in these protein classes results from wheat's hexaploid nature (Kasarda et al 1976, Bietz and Huebner 1980) and from gene duplication and mutation (Bietz et al 1977, Bietz and Wall 1980). For the storage proteins (gliadin and glutenin), large groups of homologous proteins result; since their expression is nearly invariant under different environmental and agronomic conditions, they serve as sensitive genotypic indicators.

Because of their heterogeneity, ready extractability, and relatively low (approximately 30,000 daltons) molecular weights, gliadin proteins are routinely analyzed to identify wheat varieties. Such information may indicate lots of wheat or flour suitable or unsuitable for specific applications, and may also be used in marketing, certifying, registering or breeding wheats. Electrophoresis of gliadins at acidic pH is now routinely used for varietal identification; Wrigley et al (1982) have described three of these methods in detail, and referred to numerous other variations of the method. However, electrophoresis may be subject to technical problems and experimental variability, and consequently modifications of these methods continue to be made (Tkachuk and Metlish 1980, Lookhart et al 1982, Khan et al 1983).

The introduction of large pore (≥300 Å) reversed-phase high-

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

performance liquid chromatography (RP-HPLC) columns capable of separating high molecular weight proteins promises to revolutionize many previous analytical methodologies (Regnier and Gooding 1980, Hearn et al 1982). RP-HPLC, like hydrophobic interaction chromatography on cross-linked agarose gels to which phenyl or octyl groups are attached, separates proteins on the basis of differences in surface hydrophobicity. Unlike hydrophobic interaction chromatography, however, RP-HPLC is a high-resolution technique, frequently resolving more components than alternative electrophoretic or chromatographic methods. RP-HPLC is fast, sensitive, and easily quantitated; good run-to-run reproducibility occurs, and the method is amenable to automated analysis.

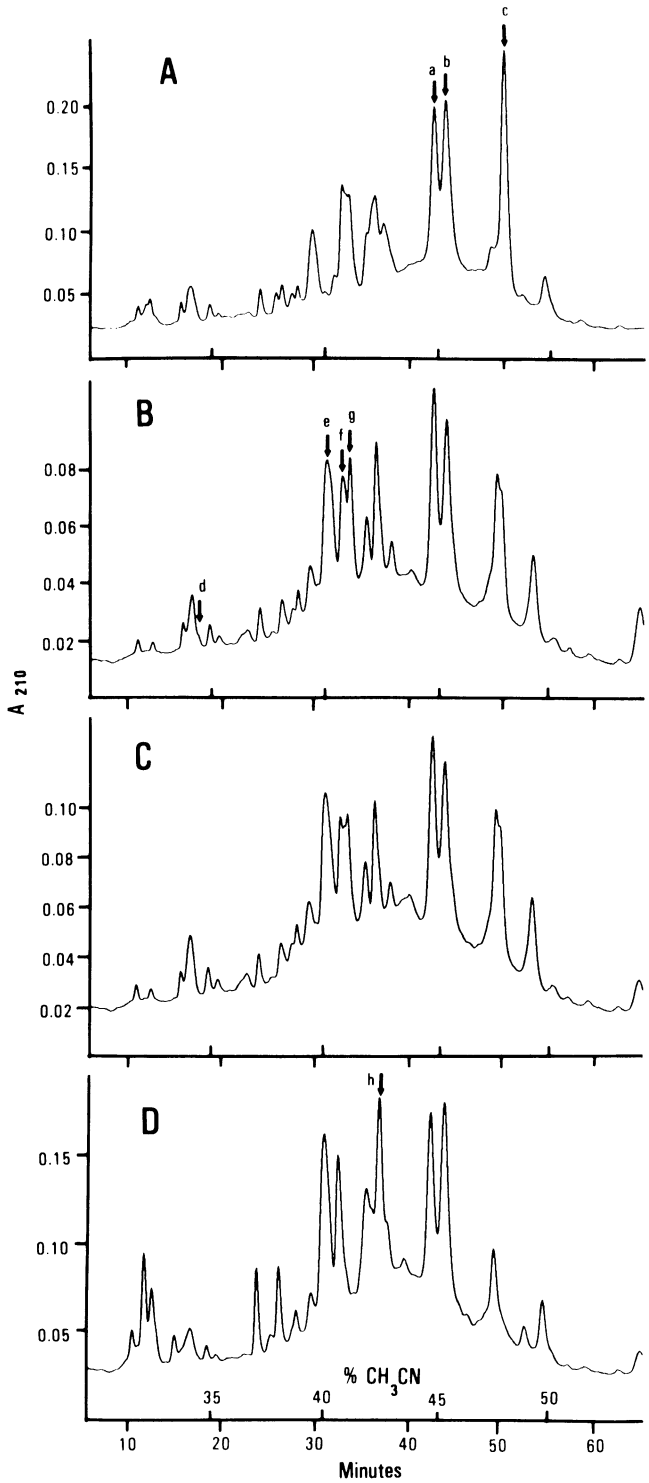

Fig. 1. RP-HPLC comparison of gliadin extracts from the hard red winter wheat varieties: **A,** Centurk; **B,** Scout 66; **C,** Homestead; and **D,** Triumph. Arrows in **A** indicate peaks a (42.39 min), b (43.61 min), and c (49.79 min); arrows in **B** indicate peaks d (17.43 min), e (31.05 min), f (32.70 min), and g (33.51 min); arrow in **D** indicates peak h (36.89 min).

Bietz (1983) first demonstrated that resolution of cereal proteins by RP-HPLC generally equals or exceeds that of other methods; for example, gliadin can be resolved into as many or more components by RP-HPLC as by one-dimensional electrophoresis. A subsequent manuscript (Bietz et al 1984) defined the optimal conditions for extraction and analysis of gliadins by RP-HPLC. The present article describes studies that demonstrate the potential of RP-HPLC for varietal identification through analysis of gliadins extracted from single kernels or small samples. In addition, we show that RP-HPLC of gliadins can indicate quality, serve as a selection tool, indicate varietal purity, differentiate wheat classes, and serve as a valuable aid in wheat breeding programs and genetic studies.

MATERIALS AND METHODS

Materials

Wheat samples used for all studies were obtained from P. Mattern, L. R. Joppa, V. Youngs, G. Rubenthaler, R. L. Clements, E. R. Sears, D. D. Kasarda, and R. Tkachuk. Solvents and chemicals used in all studies were reagent grade or better, and have been described previously in detail (Bietz 1983).

Sample Preparation

Single wheat kernels were ground with mortar and pestle; 10-kernel samples were crushed with a pulverizing mill (Paulis and Wall 1979). Ground kernels (or nondefatted flour samples) were subsequently extracted with 70% ethanol (v/v) (2.0 ml/50 mg) for approximately 30 min with continuous agitation using a Buchler Vortex-Evaporator (Bietz et al 1984); clear supernatants were obtained by centrifugation (10 min at 27,000 × *g*).

Chromatography

Methods for RP-HPLC of gliadins have been described in detail (Bietz 1983, Bietz et al 1984). Briefly, 50 μl samples were injected with a Waters WISP 710B automatic sample injector onto a 250 × 4.1 mm i.d. SynChropak RP-P (C_{18}) column. A linear 55-min gradient from 80% solvent A + 20% solvent B to 45% solvent A + 55% solvent B at 1.0 ml/min, followed by a 10-min hold at final gradient conditions, was used. It was generated with Waters M6000A and M45 delivery systems and a model 660 solvent programmer; solvent A was 15% acetonitrile (ACN) + 0.1% trifluoroacetic acid (TFA), and solvent B was 80% ACN + 0.1% TFA. Eluted components were detected at 210 nm with a Waters model 450 variable-wavelength detector, and data were recorded on a Houston Instruments Omniscribe recorder (0.2 AUFS/10 mV) and simultaneously stored in a ModComp computer system for subsequent integration and replotting.

RESULTS AND DISCUSSION

Wheat Varietal Identification

By RP-HPLC, we have now examined gliadins extracted from several hundred wheat genotypes, including most major commonly grown varieties. The following sections, presenting representative results for each major U.S. wheat class, demonstrate that most varieties, even those having very similar pedigrees, can be readily differentiated and thereby identified through RP-HPLC.

In many wheat samples, if not all, some genetic heterogeneity exists. These off-types or alternate genotypes, sometimes called biotypes (Wrigley and Shepherd 1974, Kosmolak 1979), may have several origins (Appleyard et al 1979). The frequency of these off-types is generally minor. Nevertheless, if single kernels are analyzed there is a possibility that a nonrepresentative genotype may be chosen. Similarly, slight quantitative variability exists between samples or kernels even for homogeneous genotypes. Consequently, in any wheat varietal identification, whether by electrophoresis or by RP-HPLC, it is mandatory that either multiple single kernels or a composite sample be analyzed,

preferably from more than one source, as a standard for each variety to eliminate sample-to-sample variability. Subsequent analyses of unknowns could then be based either on single kernels or on bulk samples, but minor quantitative and qualitative differences should, until their significance is established, be regarded with caution, as in any other technique. The chromatograms presented here either represent extracts of single kernels that appear representative of a variety or represent extracts of 10-kernel samples or of flour.

Hard red winter wheats. Figure 1 presents RP-HPLC chromatograms of gliadins extracted from the hard red winter wheat varieties Centurk, Scout 66, Homestead, and Triumph, chosen here to be representative of a much larger number of varieties examined. Approximately 30–40 components are revealed from these chromatograms, including those indicated by minor peaks and shoulders. In comparison, one-dimensional electrophoresis generally separates gliadin into 20–30 components (Wrigley et al 1982), and two-dimensional electrophoresis separated gliadin from one variety into 46 components (Wrigley and Shepherd 1973). Even though a few early-eluting components in RP-HPLC chromatograms of ethanol extracts of wheats may be albumins and globulins (Bietz 1983, Bietz et al, 1984), resolution of gliadin by RP-HPLC apparently is at least as good as that achieved by one-dimensional electrophoresis. It is also interesting to note that chromatograms of all hard red winter wheat varieties we have examined contain two prominent peaks (a and b in Fig. 1A). Subsequent analyses show that these peaks are absent in durums and may therefore be characteristic of the D genome. These peaks could serve as useful internal standards to facilitate comparisons between chromatograms.

Numerous qualitative and quantitative differences permit differentiation of these (Fig. 1) and most other hard red winter wheat varieties we have examined. Among these chromatograms, for example, Centurk lacks peak e, which is present in other varieties (Fig. 1B), and has a unique or greatly increased amount of component c (Fig. 1A). Triumph has a very large peak h (Fig. 1D) and lacks peak g, which is present in the other varieties. The patterns for Scout 66 (Fig. 1B) and Homestead (Fig. 1C) are very similar but exhibit a minor but reproducible quantitative difference in the region f–g (Fig. 1B), and the small shoulder d is unique to Scout 66 (see Fig. 1B).

Obviously, very small quantitative and qualitative differences must be used with caution in differentiating varieties. More importantly, marked similarity between RP-HPLC patterns, as observed for Scout 66 and Homestead, may reflect similar pedigrees, in this case the contribution of the variety Scout (Zeven and Zeven-Hissink 1976). In wheats having similar pedigrees, it is not suprising to find similar gliadin compositions, especially since genes coding gliadins are located only on chromosomes 1 and 6 (Shepherd 1968), whereas major genes differentiating varieties could well be located elsewhere.

Our results suggest that quantitative differences between varieties may in some cases be more apparent by RP-HPLC than by electrophoresis, especially when densitometry is not performed. Major quantitative differences may indicate unresolved components of some peaks, since gliadin's actual heterogeneity may be considerably greater than the number of peaks resolved. Minor quantitative differences, however, may indicate normal sample-to-sample variability due to environment, conditions of growth, or degree of maturity. Additional studies are in progress to better evaluate environmental variability within a genotype, as well as to examine genetically similar varieties. We believe that careful control of experimental conditions in RP-HPLC will permit differentiation of many varieties that are genetically similar on the basis of minor but significant chromatographic differences.

Hard red spring wheats. Figure 2 presents RP-HPLC results for gliadins extracted from the hard red spring wheat varieties Butte, Olaf, Anza, Era, and Solar. RP-HPLC can differentiate and thereby identify these and most other varieties by quantitative and qualitative differences in numerous peaks. For example, of these five varieties only Butte, Olaf, and Anza (Fig. 2, A–C) have the major peak a; Butte (Fig. 2A) has peak b, which is absent in the other two, and Anza (Fig. 2C) has peak c, which Olaf lacks.

Era and Solar are very similar, but minor differences, such as peaks e and f in Solar (Fig. 2E) and peak d in Era (Fig. 2D), were observed between these two varieties. These differences were confirmed in samples from an independent source, suggesting that RP-HPLC may be able to differentiate some varieties that give identical electrophoresis patterns (as do Era and Solar, data not shown; also Jones et al 1982). Analysis of additional samples would

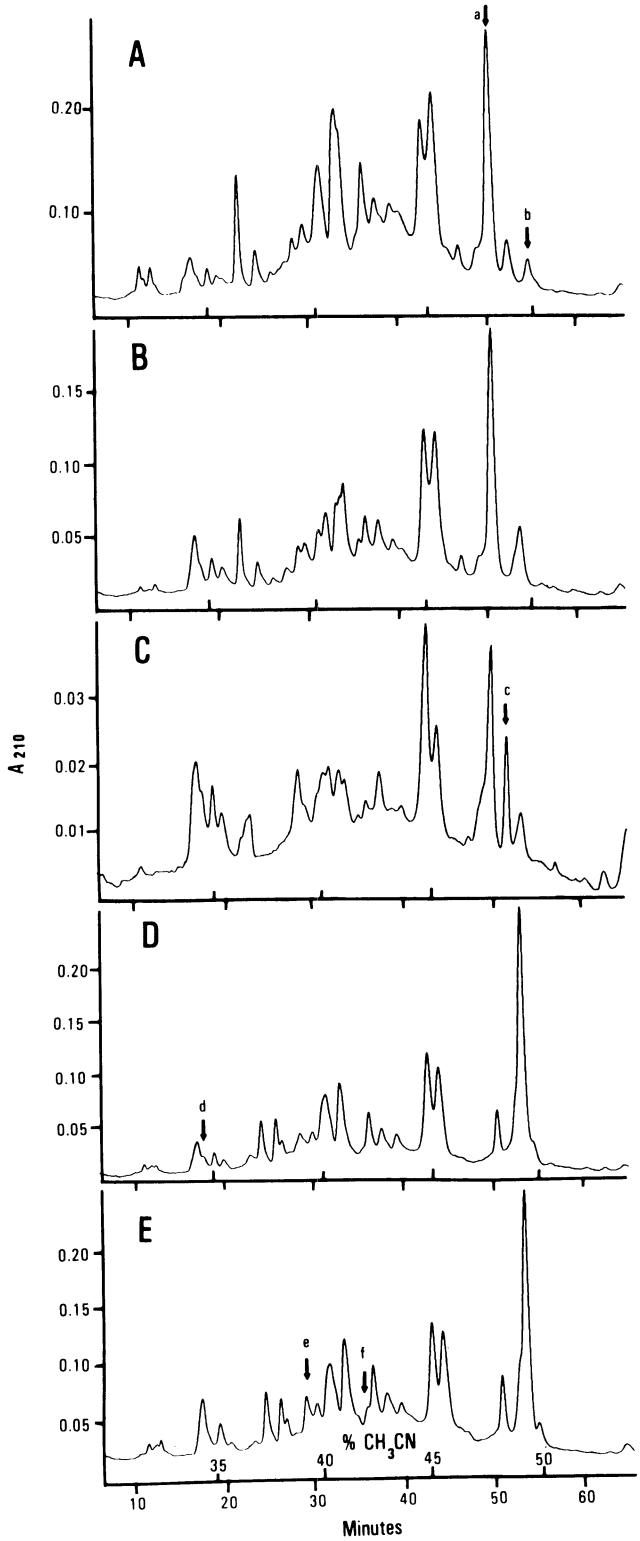

Fig. 2. RP-HPLC comparison of gliadin extracts from the hard red spring wheat varieties: **A,** Butte; **B,** Olaf; **C,** Anza; **D,** Era; and **E,** Solar. Arrows in **A** indicate peaks a (49.80 min) and b (54.16 min); arrow in **C** indicates peak c (51.41 min); arrow in **D** indicates peak d (17.66 min); and arrows in **E** indicate peaks e (28.44 min), and f (35.25 min).

be necessary, however, to be certain of the significance of such minor differences. The reported pedigrees of Era and Solar differ considerably (Zeven and Zeven-Hissink 1976, Jones et al 1982), so our results also support the suggestion (Jones et al 1982) that one or both reported pedigrees for these two varieties are probably in error.

Soft red winter wheats. RP-HPLC chromatograms of gliadins from three common soft red winter wheats, Oasis, Arthur 71, and Doublecrop, are shown in Fig. 3. As noted by electrophoresis (Jones et al 1982), these and other soft red winter wheat varieties have very similar gliadin compositions, due to similar pedigrees. Oasis (Fig. 3A) and Arthur 71 (Fig. 3B) could not be distinguished by electrophoresis, but RP-HPLC reveals quantitative differences in peaks a and b (Fig. 3A) that may differentiate these varieties. Doublecrop (Fig. 3C) differs from the other two varieties in having the unique component d.

White wheats. RP-HPLC chromatograms of gliadins from the white wheat varieties Hyslop, McDermid, Stephens, and Fielder are shown in Fig. 4. There are numerous quantitative and qualitative differences among these varieties. For example, McDermid (Fig. 4B) lacks the major peak a present in the other varieties (Fig. 4A). Fielder (Fig. 4D) lacks the major peak b that is present in Stephens (Fig. 4C) and Hyslop (Fig. 4A). Stephens (Fig. 4C) and Hyslop (Fig. 4A) are easily differentiated by peak c in Hyslop (Fig. 4A). Similarly, all other white wheats examined (Daws, Luke, Nugaines, Sprague, Fieldwin, Marfed, Twin, Moro, and Yorkstar) could be differentiated, with the exception of Fieldwin and Fielder (Fig. 4D), which had essentially identical RP-HPLC patterns. This is hardly surprising, since Fielder and Fieldwin have identical pedigrees (Zeven and Zeven-Hissink 1976) and were selected from the same cross. Jones et al (1982) found slight differences in electrophoregrams of these varieties that may permit identification of an unknown by comparison to standards run on the same gel. We examined numerous single kernels of Fielder and Fieldwin, and found a few atypical kernels, having gliadin compositions markedly different from the major genotype, in each variety. Different off-types were found in each variety. If such differing off-types are consistently present in the varieties, analysis of flours from bulk samples might indicate a slight difference in gliadin composition, as observed by electrophoresis.

Durum wheats. Figure 5 presents RP-HPLC patterns of gliadins from the durum varieties Ward, Rolette, Edmore, and Mexicali. These and all other durum varieties examined, including Produra, Leeds, Langdon, and Cando, could all be differentiated on the basis of their chromatographic patterns (although differences between Ward and Langdon, which have similar pedigrees, were mainly quantitative rather than qualitative). For example, in Fig. 5, two distinct types of patterns occur: Ward (Fig. 5A) and Rolette (Fig. 5B) have a characteristic quartet of peaks (b, c, d, and e in Fig. 5A),

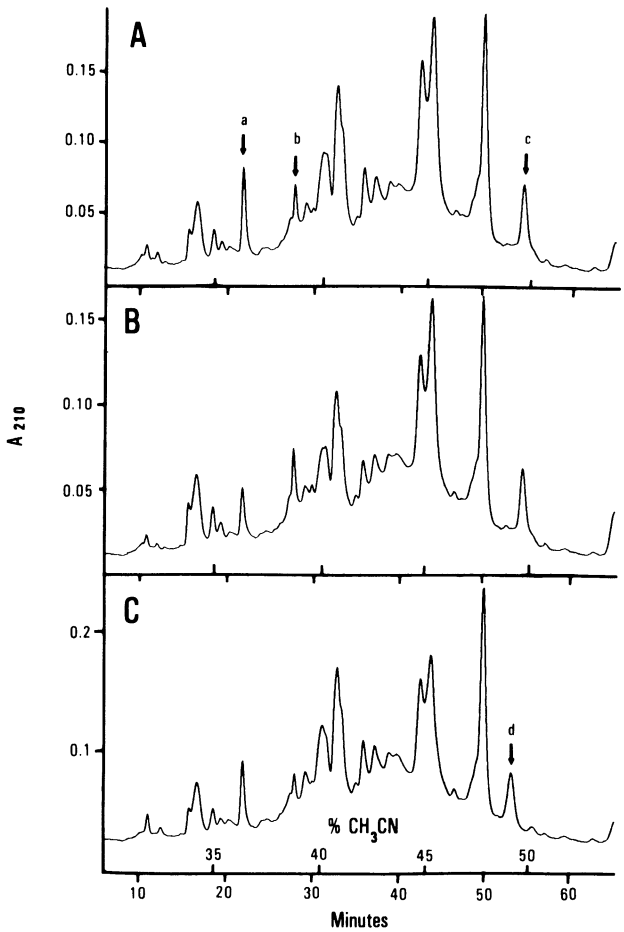

Fig. 3. RP-HPLC of gliadins extracted from the soft red winter wheat varieties: **A,** Oasis; **B,** Arthur 71; and **C,** Doublecrop. Arrows in **A** denote peaks a (21.89 min), b (27.79 min), and c (54.14 min); arrow in **C** indicates peak d (52.90 min).

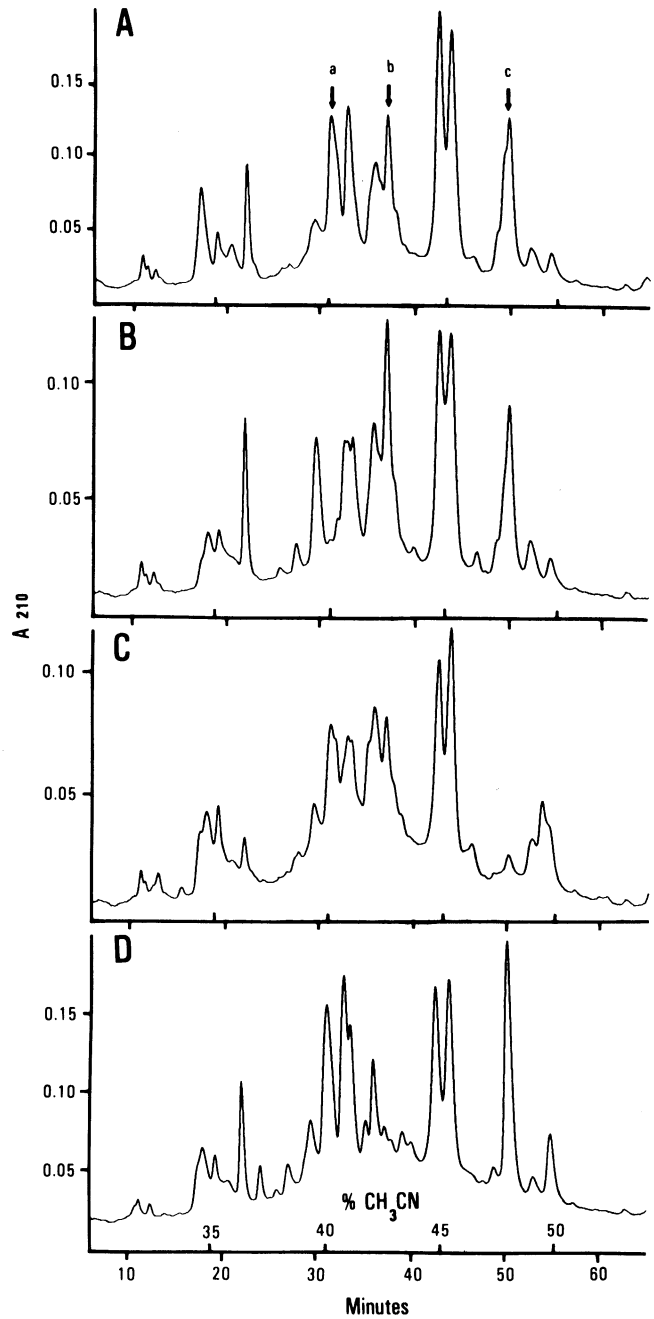

Fig. 4. RP-HPLC of gliadins from the white wheat varieties: **A,** Hyslop; **B,** McDermid; **C,** Stephens; and **D,** Fielder. Arrows in **A** denote peaks a (30.97 min), b (36.96 min), and c (49.77 min).

but are easily differentiated by peak a (Fig. 5A). Edmore (Fig. 5C) and Mexicali (Fig. 5D) both contain major peaks i and j (Fig. 5C), but are easily differentiated by numerous components, including peaks f, g, and h in Edmore (Fig. 5C).

Comparison of RP-HPLC patterns of tetraploid durum wheats (Fig. 5) to those of hexaploid wheats (Figs. 1–4) clearly shows that durums lack the pair of major peaks (a and b in Fig. 1A) that are characteristic of all examined hexaploid wheats. Our results also demonstrate significant genetic diversity among durum wheats; in contrast, electrophoregrams of many durum cultivars were quite similar (Jones et al 1982), although most could be differentiated. Minor structural differences in durum gliadins may produce major differences in surface hydrophobicities, permitting ready differentiation by RP-HPLC, but may have little effect on ionic characteristics. Because of their complementary nature, RP-HPLC and electrophoresis of gliadins together may differentiate varieties having similar pedigrees.

Through characterization of gliadins extracted from numerous additional durum varieties, we have observed that all varieties examined either have peaks b, c, d, and e (as in Fig. 5A) or they have peaks i and j (Fig. 5C). This division of durum cultivars is identical to that based on gliadin electrophoresis bands 45 and 42 (Damidaux et al 1978), which are predictors of durum gluten strength and weakness, respectively. Burnouf and Bietz (1984) recently described these studies in detail and demonstrated that electrophoresis band 45 corresponds to peak i (Fig. 5C) and that band 42 corresponds to peak b (Fig. 5A).

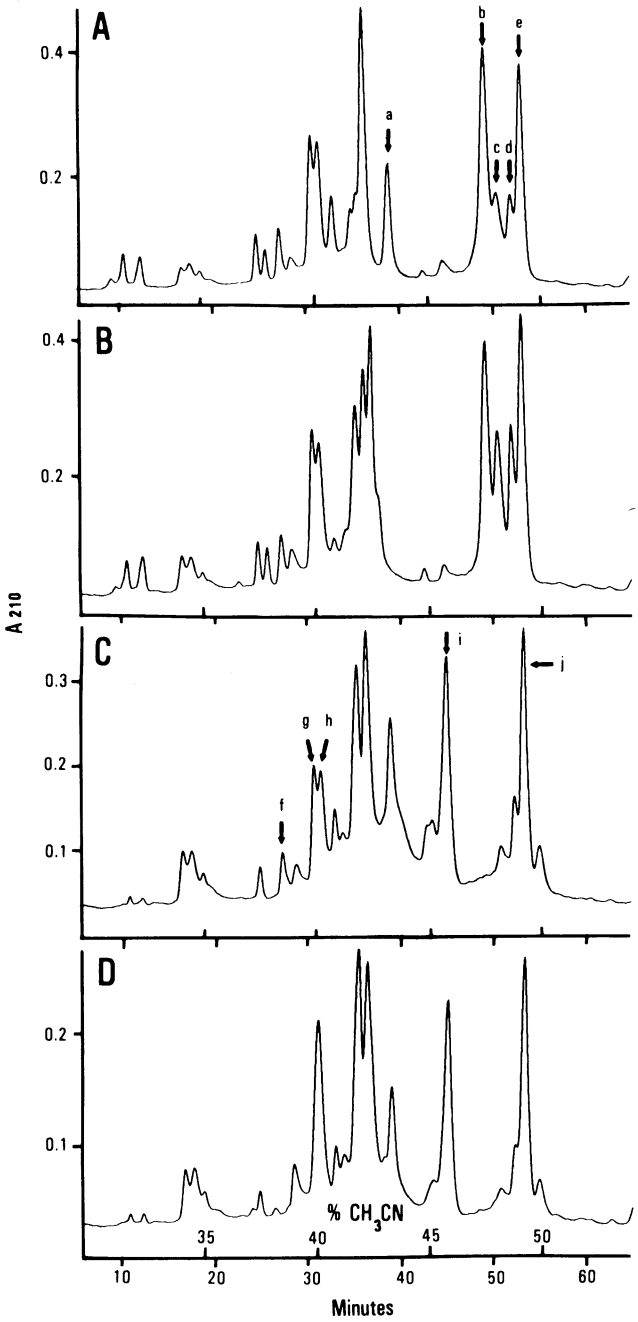

Fig. 5. RP-HPLC patterns of gliadins extracted from the durum wheat varieties: **A,** Ward; **B,** Rolette; **C,** Edmore; and **D,** Mexicali. Arrows in **A** indicate components a (39.02 min), b (49.30 min), c (50.56 min), d (52.04 min), and e (53.15 min); arrows in **C** indicate peaks f (27.59 min), g (31.00 min), h (31.71 min), i (45.12 min), and j (53.35 min).

RP-HPLC of Wheat Aneuploids

Because each gliadin polypeptide is coded by a specific gene, electrophoresis of proteins isolated from wheat aneuploids, which have atypical chromosomal contents, has enabled researchers to pinpoint the chromosomal location of these genes (Shepherd 1968, Wrigley and Shepherd 1973, Brown and Flavell 1981). Because specific gliadin components can either directly affect wheat quality characteristics or be closely linked with other genes having desirable or undesirable effects (Wrigley et al 1982), electrophoresis of gliadins may be valuable in breeding and selection.

RP-HPLC is also a powerful tool for analysis of wheat aneuploids, as shown in Fig. 6. Ditelocentric lines contain only the indicated arms of the specified chromosome, and lack the other arm; eg, ditelo $1A_L$ contains the long arms of both 1A

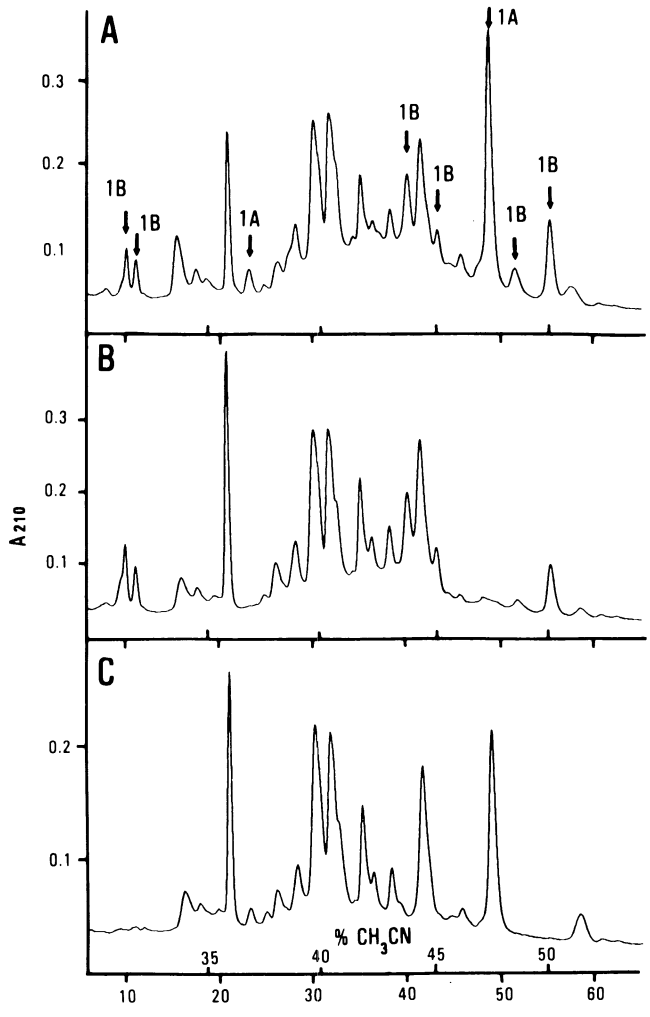

Fig. 6. RP-HPLC analysis of gliadins extracted from: **A,** Chinese Spring; **B,** Chinese Spring ditelo $1A_L$; and **C,** Chinese Spring ditelo $1B_L$. Arrows and chromosome designations in **A** indicate peaks absent in corresponding ditelo lines.

chromosomes, and lacks the short arms (which are known to code gliadin proteins) (Shepherd 1968). Thus, chromatograms of ditelo lines lack those peaks of their parent variety that correspond to gliadins coded by genes located on the missing chromosome arm. The data of Fig. 6 identify (as indicated in Fig. 6A) eight components coded by genes on the short arms of chromosomes 1A and 1B.

Additional studies are in progress to establish the genetic control of all hexaploid wheat gliadin peaks resolved by RP-HPLC, to extend these studies to durum wheat aneuploids, and to establish the chromosomal control of glutenin subunit peaks. Specific wheat chromosomes are known to be associated with desirable quality characteristics, and to contain genes coding gliadin and glutenin polypeptides related to quality. Identification of such proteins in RP-HPLC patterns should enable RP-HPLC to be used for quality determinations.

RP-HPLC as a Selection Tool

In any breeding program, genetic diversity must occur to permit improvement, and selection or rejection of lines must depend upon specific phenotypic or genetic characteristics. Since gliadins are excellent genotypic indicators, gliadin electrophoresis is a useful selection tool; it can demonstrate varietal purity, aid in registration of varieties, and detect off-types. Our studies demonstrate that RP-HPLC analysis of gliadins can serve the same purposes. An example is shown in Fig. 7.

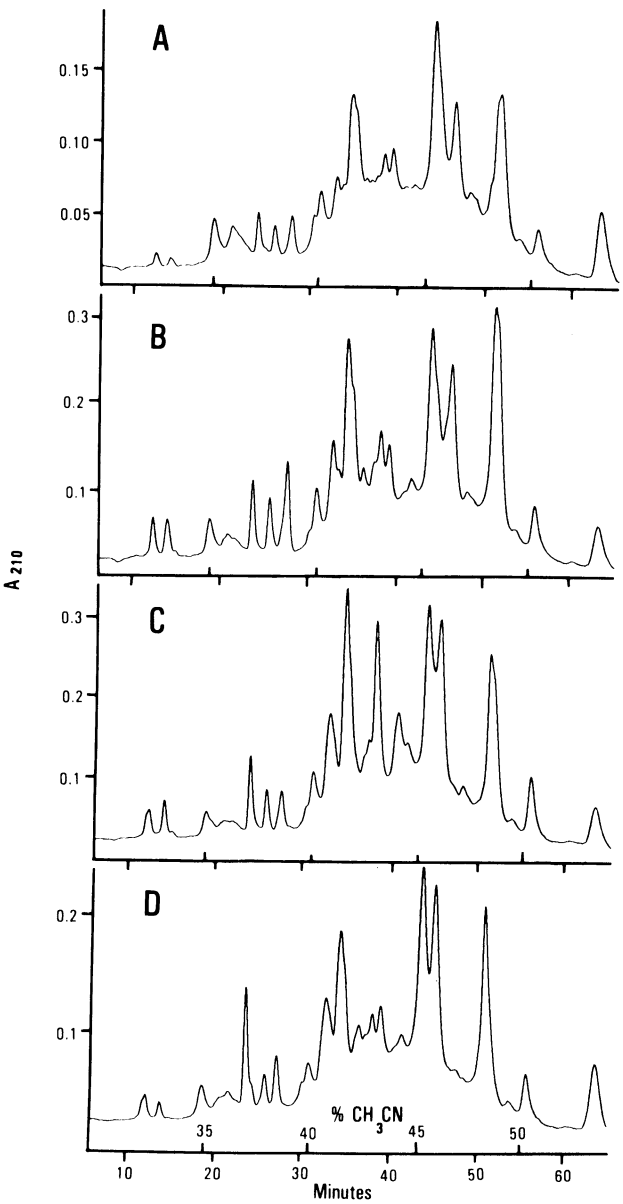

Fig. 7. RP-HPLC analysis of gliadins extracted from four individual kernels of the hexaploid wheat Nap Hal.

Nap Hal is a hexaploid wheat of considerable interest in hard red winter wheat breeding because it carries genes that promote both elevated protein and elevated lysine (Johnson et al 1978). Using electrophoresis, Bietz et al (1975) previously noted that Nap Hal has a highly unusual glutenin subunit composition and also contains numerous genotypes. We therefore selected 12 kernels of Nap Hal that differed in size and surface characteristics and analyzed their extracted gliadins by RP-HPLC. At least four major genotypes were present (Fig. 7), as indicated by major qualitative and quantitative differences among the resulting chromatograms. These results demonstrate that it may be desirable to identify or authenticate the identity of varieties used in test crosses, especially since Jones et al (1982) showed that 4% of samples received from breeders and germplasm storage banks were mislabeled. Different Nap Hal off-types, for example, may have very different properties. These results demonstrate that RP-HPLC of gliadins is a useful selection tool in wheat breeding and improvement.

CONCLUSIONS

Our results clearly demonstrate that RP-HPLC of gliadins extracted from wheat kernels or flour samples can differentiate, and thereby serve to identify, most wheat varieties. Only varieties that have very similar pedigrees, for which variability among gliadins is slight or nonexistent, give similar or identical chromatographic patterns. RP-HPLC can thus estimate genetic relatedness of varieties, as well as identify them. RP-HPLC is also a new and valuable method for selection, for demonstration of off-types and in screening for desirable quality characteristics.

RP-HPLC separates gliadins on the basis of differences in surface hydrophobicity, a characteristic different from those used in most other chromatographic and electrophoretic techniques. RP-HPLC therefore complements these other methods by providing an additional way to examine samples.

Some potential uses of RP-HPLC and electrophoresis are similar, so some comparison of the methods may be warranted. Electrophoresis is both slow and fast: one to two days may be required for analysis, but numerous samples can be processed simultaneously, so its potential for routine use is high. Equipment for electrophoresis is fairly simple and inexpensive, but results may vary with reagent purity, polymerization conditions, temperature factors, nonuniform electric fields, and staining and destaining methods; electrophoresis is also labor intensive. In some respects, successful electrophoresis requires both good science and good art: many small, nearly indefinable variables exist that can deleteriously affect final results. However, careful adherence to uniformity and optimization of all experimental conditions (Lookhart et al 1982, Khan et al 1983) may assure good results. Samples and standards can also be simultaneously compared by electrophoresis.

RP-HPLC also has both disadvantages and advantages. Equipment is more costly and complex, but techniques are relatively simple and require, in our opinion, less "art" than does electrophoresis. Analyses can be totally automated, so relatively little operator time is required. The method is rapid, compared to electrophoresis, in that an unknown sample can presently be analyzed in about 1.5 hr; nevertheless, only about 20 analyses per instrument per day can be performed using today's technology. Alterations in columns and techniques may, however, significantly increase sample throughput; for example, Burnouf and Bietz (1984) were able to determine durum gluten quality in 5–10 min by such procedures. Some variability in solvent purity, column stability, flow rate, gradient formation, and technical operation may exist in RP-HPLC, but we believe that these factors are readily controllable. As with any new method, improved results in RP-HPLC can be anticipated as chromatographic conditions and columns are optimized; studies are now in progress, for example, to determine which RP-HPLC columns give the best separations of

cereal proteins and to examine variability among numerous columns of the same type (Bietz et al 1983).

The real advantage of RP-HPLC is undoubtedly that it complements other techniques. Resolution of RP-HPLC generally equals or exceeds that achieved by other methods, and reproducibility of retention times (relative standard deviation, 0.3295) and overall chromatographic patterns is excellent (Bietz 1983). As with electrophoresis, sample-to-sample variability must be considered in evaluating all results. The ability to detect proteins at 210 nm on the basis of peptide bonds, rather than by using stains that react differently with different proteins, is a major advantage in RP-HPLC (although numerous other compounds also absorb at this wavelength). Quantitative information resulting from RP-HPLC can therefore be accurately and automatically obtained, permitting the use of numerous computer-assisted techniques to process, compare, and store data.

One major problem with any high-resolution technique is that much more qualitative and quantitative information is generated than can be readily comprehended and utilized. At least three computer-assisted techniques have been devised to deal with this problem in the use of electrophoresis of wheat proteins for varietal identification (Bushuk et al 1978, Wrigley et al 1982, Jones et al 1982, Lookhart et al 1983). Nevertheless, some subjective interpretation of quantitative electrophoresis data is often necessary. In RP-HPLC, however, accurate elution times and peak areas may be easily obtained using fairly simple integrator or computer systems. It should be possible to automatically store and compare such data sets through the use of a computer; for example, unknown varieties might be identified by comparison to a stored library of standard chromatographic data, applying corrections based upon elution times of sample constituents or of internal standards added to samples. In addition, computer-assisted examination of RP-HPLC chromatographic data probably will reveal specific components indicative of milling or baking quality or correlated with other specific characteristics.

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