Improved Procedures for Rapid Wheat Varietal Identification by Reversed-Phase High-Performance Liquid Chromatography of Gliadin

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ABSTRACT

Wheat varieties may be identified through reversed-phase high-performance liquid chromatography (RP-HPLC) of gliadins. RP-HPLC is fast, reproducible, and gives good resolution, but improvements are still possible by altering run time, flow rate, and temperature, and by using internal or external standards. Rapid gradients (10–15 min, 2.5–3.0 ml/min) gave less resolution than longer (about one hour) runs, but still differentiated most varieties, permitting more than 100 analyses per day per instrument. Reproducibility was improved by substituting H_2O and CH_3CN (+0.1% trifluoroacetic acid) for H_2O/CH_3CN/trifluoroacetic acid solvent blends used earlier. Constant temperature is essential for maximum reproducibility, and resolution is significantly better at elevated (70°C) temperature, possibly caused by better dissociation of proteins. Alkylphenones (C_2–C_{10}) may serve as standards to eliminate intra- and interlaboratory variability that arises from different experimental conditions. A hydrophobicity index is proposed to characterize proteins in relation to standards rather than by elution time. These modifications for improving speed, resolution, and reproducibility of RP-HPLC gliadin analysis may permit automated wheat varietal identification by computer, free of subjective data evaluation.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a fast, reproducible, high-resolution technique for separation of cereal proteins (Bietz 1983). Initial RP-HPLC studies of wheat varietal identification (Bietz et al 1984a,b) were performed at 30°C using mixed solvents containing H_2O, CH_3CN, and trifluoroacetic acid (TFA). A total of about one hour was required per analysis, permitting about 20 analyses a day per instrument; although rapid, this time may be too slow for some applications. In addition, although RP-HPLC resolution and reproducibility were excellent within any series of runs, changes in columns, solvents, or other conditions could affect elution times, making some comparisons difficult.

We here report effects of gradient conditions, temperature, and the use of standards on wheat varietal identification by RP-HPLC. Improvements in speed, resolution, and reproducibility make RP-HPLC more useful and indicate that automated varietal identification may be possible.

MATERIALS AND METHODS

Materials

HPLC-grade CH_3CN and Sequanal-grade TFA (Pierce Chemical Co., Rockford, IL) were used as described previously (Bietz 1983; Bietz et al 1984a,b). Water was purified with a Barnstead NANOpure system. Alkylphenone standards were from Pierce. All other chemicals were reagent grade or better. J. R. S. Ellis (High Wycombe, U.K.) generously supplied samples of commonly marketed English wheat varieties Armada, Avalon, Fenman, Flanders, Galahad, Longbow, Mardler, Mission, Norman, and Rapier. Sources of the hard red winter wheat varieties Centurk, Arkan, Sage, Newton, and Wanser, the spring wheat varieties Chinese Spring and Anza, and the durum variety Langdon used in this study were described previously (Bietz et al 1984b). Zein from maize inbred W64A was generously supplied by J. W. Paulis (this laboratory).

Apparatus

RP-HPLC was performed with SynChropak RP-P (C_{18}) (250 × 4.1 mm i.d.), Brownlee Aquapore RP-300 (C_8) 250 × 4.1 mm i.d.), and Beckman Ultrapore RPSC (75 × 4.6 mm i.d.) columns and apparatus described previously (Bietz 1983; Bietz et al 1984a,b). For rapid analyses, columns such as the Brownlee and Beckman, which each operate at about 300 psi at 1.0 ml/min, permit increased flow rates without generating unduly high pressure. Outgassing of solvents in the detector was prevented by attaching about 1 m of capillary Teflon tubing to the flowcell outlet.

Experimental Procedures

Gliadins were extracted and analyzed by RP-HPLC as described previously (Bietz 1983; Bietz et al 1984a,b) except for indicated changes in time, temperature, or gradient conditions. Gradient times were varied from approximately 60 min, as in the normal procedure, to 8 min. For rapid analyses, the flow rate was 2.5–3.0 ml/min, and final gradient conditions were maintained for 2 min. Column temperatures were varied from 15 to 70°C; temperatures above 70°C were not tested because of the volatility of CH_3CN.

A re-equilibration time of 3 min sufficed between runs using the rapid procedure. Although longer equilibration times have been suggested (Dolan et al 1979), we have found that a 3-min re-equilibration (equivalent to approximately 5 column volumes, as is used in longer gradient procedures [Bietz et al 1984a,b]) provides excellent reproducibility (see below). It is possible that variability in early portions of chromatograms is greater than that for late-eluting peaks, but gradient conditions are chosen to ensure that most major components do not elute close to the solvent peak. Furthermore, the constant analytical conditions achieved using an automated chromatographic system provide constant, even if not total, column regeneration, thus maximizing reproducibility throughout and between chromatograms.

Alkylphenone mixtures were evaluated as internal and external RP-HPLC standards. For 8–15 min gradient runs, 4 μl each of acetophenone (C_2), propiophenone (C_3), n-butyrophenone (C_4), and valerophenone (C_5), added to 5 mg of hexanophenone (C_6), was dissolved in 25 ml CH_3CN + 0.1% TFA, and 4 μl of the resulting solution was analyzed; alternatively, 5 μl of the mixture was added per 200 μl of sample as an internal standard. For 50–60 min analyses, 4 μl each of C_2, C_3, C_4, C_5, and octanophenone (C_8), plus 3 mg of C_6 and decanophenone (C_{10}), were dissolved in 25 ml of CH_3CN + 0.1% TFA, and analyzed as above.

RESULTS AND DISCUSSION

In separate experiments, we investigated effects of solvent composition, the use of internal and external standards, the necessity of maintaining constant column temperature, and the effect of elevated column temperature. The following sections describe these studies, and the resulting improvements in wheat varietal identification by RP-HPLC.

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Effects of Solvent Composition

In initial RP-HPLC procedures for wheat varietal identification (Bietz et al 1984a,b), gradients were generated from 15% aqueous CH_3CN + 0.1% TFA (solvent A) and 80% aqueous CH_3CN + 0.1% TFA (solvent B), which covered the entire hydrophobicity range of cereal proteins (Bietz 1983) and minimized outgassing in detector flowcells. For most purposes run-to-run reproducibility was satisfactory; upon replicate analyses, however, a slow but steady increase in elution time was noted for any peak; typical results are shown in Figure 1. The changes were not major (a few hundredths of a minute), but may be excessive for automated peak recognition and identification. We postulated that increased elution times result from changing solvent composition caused by CH_3CN evaporation from the mixed solvents being used.

Consequently, we tested pure H_2O (+ 0.1% TFA) as solvent A, and CH_3CN (+ 0.1% TFA) as solvent B: selective evaporation from these mixtures would only slightly alter the TFA concentration, having relatively little effect on final results (Bietz 1983, Huebner and Bietz 1984). Typical results for 9 peaks from 27 consecutive replicate analyses of Arkan gliadin, using a 25–50% B linear gradient over 8 min at 2.5 ml/min, with a total run time of 12 min and an equilibration delay of 3 min, are shown in Table I. Nearly constant elution times were now achieved: for example, the elution time for peak 10 varied from 8.00–8.03 min, and the average coefficient of variation for these data was 0.0942. Thus, these modified solvent conditions should improve RP-HPLC reproducibility for wheat varietal identification. We have observed, as did Marchylo and Kruger (1984), that reproducibility for successive analyses is better than that for analyses carried out over longer time periods, most likely because instrument and column performance vary slightly, even though solvent conditions remain constant. The use of standards (see below) could alleviate this problem.

Marchylo and Kruger (1984) also noted, in RP-HPLC studies of barley varietal identification, that retention times vary as a result of CH_3CN evaporation from mixed solvents. They minimized this problem by using sealed solvent reservoirs; however, we think that using H_2O + 0.1% TFA and CH_3CN + 0.1% TFA as solvents is a simpler and more general solution to the problem.

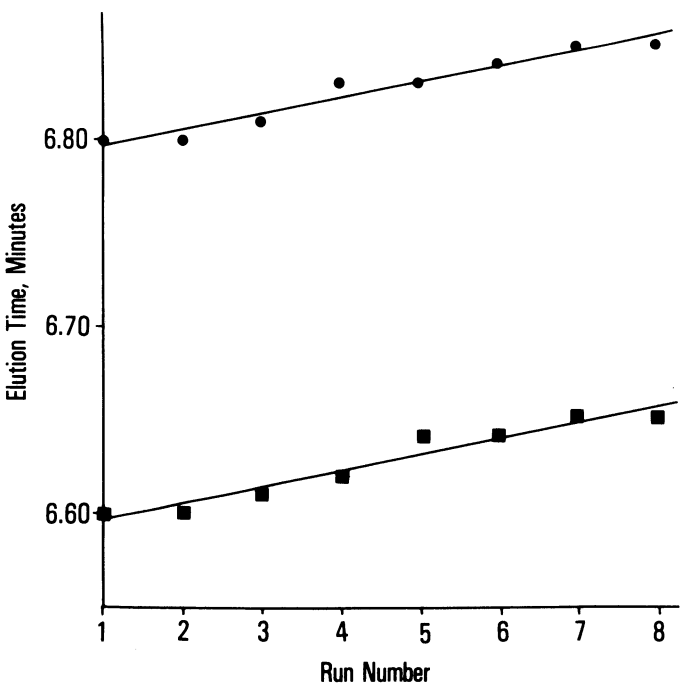

Fig. 1. Effect of evaporation from mixed solvents on RP-HPLC elution times. A sample of Centurk gliadin was repeatedly analyzed on a Brownlee RP-300 column at 2.5 ml/min using a gradient from 15–55% solvent B/8 min, with a total run time of 12 min and a 3-min re-equilibration between runs. Solvents used were 15% CH_3CN + 0.1% TFA (solvent A) and 80% CH_3CN + 0.1% TFA (solvent B). Elution times of two characteristic peaks were plotted for eight identical consecutive runs. Least squares regression analysis yielded the indicated straight lines (correlation coefficients both = 0.97), showing increasing elution time with increased solvent age.

Use of Standards in RP-HPLC Wheat Varietal Identification

Surface hydrophobicity of proteins should remain relatively constant in a given solvent, but RP-HPLC elution times may vary with column, solvents, or temperature. To minimize such variability and improve reproducibility and the ability to identify

TABLE I
Precision of RP-HPLC Data Using Solvent A = H_2O + 0.1% Trifluoroacetic Acid (TFA) and Solvent B = CH_3CN + 0.1% TFA

Peak	Mean Elution Time[a] (min)	Standard Deviation	Coefficient of Variation[b]
2	5.561	0.0261	0.4697
3	6.697	0.0166	0.2478
4	6.994	0.0069	0.0992
6	7.304	0.0079	0.1084
10	8.009	0.0089	0.1113
13	8.369	0.0060	0.0719
16	9.240	0.0052	0.0560
18	9.829	0.0058	0.0587
19	10.150	0.0000	0.0000
		Mean	0.0942

[a]Determined for 27 replicate analyses.
[b]Coefficient of variation equals standard deviation divided by mean, times 100.

TABLE II
Effect of Constant Temperature on Precision of RP-HPLC Data for Gliadins

Series	Temperature	Run Time (min)	Replicates	Peaks	Average Coefficient of Variation
1	Ambient	8	12	5	0.2817
2	Ambient	8	9	5	0.9629
3	70°C	8	46	3	0.1391
4	70°C	55	10	6	0.1281

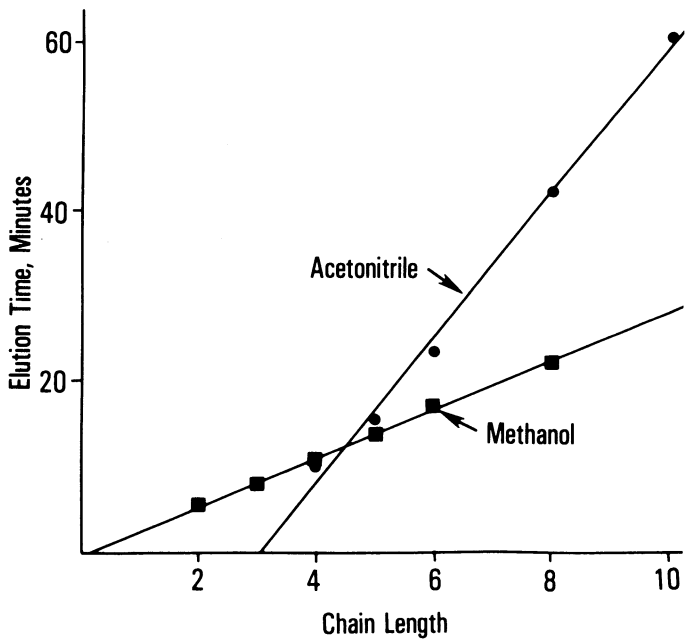

Fig. 2. Linear relationships (correlation coefficients both = 0.99) between side-chain length of alkylphenone internal standards and elution time in linear gradients of CH_3CN and methanol.

specific components, it should be possible to separately analyze known compounds (external standards) or to include them with samples to be analyzed (internal standards). Alkylphenones varying in carbon side-chain length have been advocated as suitable RP-HPLC standards (Kikta and Stange 1977), even under gradient conditions (Pierce 1984), to monitor the performance of an HPLC system.

Available alkylphenones vary widely in hydrophobicity. In addition, we found their elution times to be linearly related to carbon side-chain length (Fig. 2), for both methanol (data from Pierce 1984) and CH_3CN (data from Fig. 3) linear gradients. These results suggest that gliadin proteins may be identified relative to alkylphenone chain length, rather than in terms of elution time or CH_3CN concentration.

Figure 3 shows an example of the potential use of alkylphenones as internal RP-HPLC standards for gliadin. When an appropriate ratio of standards to sample is used, both sample and standard peaks may be seen (some peaks in complex samples may, of course, be masked). If standard peaks are identified by side-chain length (e.g., 4.00, 5.00, 6.00, ...), elution times for sample peaks eluting between standards may be expressed as "hydrophobicity indices," determined from the proportionality of standard chain length to elution time; examples for sample peaks are noted in Figure 3. Preliminary results suggest that hydrophobicity indices are relatively independent of normal variability in experimental conditions. In many laboratory situations, at relatively constant conditions, internal standards may be unnecessary. However, it may still be desirable to analyze these or other standards periodically to test system performance.

We have only used standards to monitor peak position, and not for quantitation, which may be another real benefit (Kikta and Stange 1977). Sample quantitation would, of course, be affected by inclusion of standards in a sample; thus, for any automated identification system, where both qualitative and quantitative RP-HPLC data are used, it may be more desirable to periodically analyze standards separately from samples to normalize elution times, and as a test of system performance. Further studies are necessary to determine the applicability of these or other standards

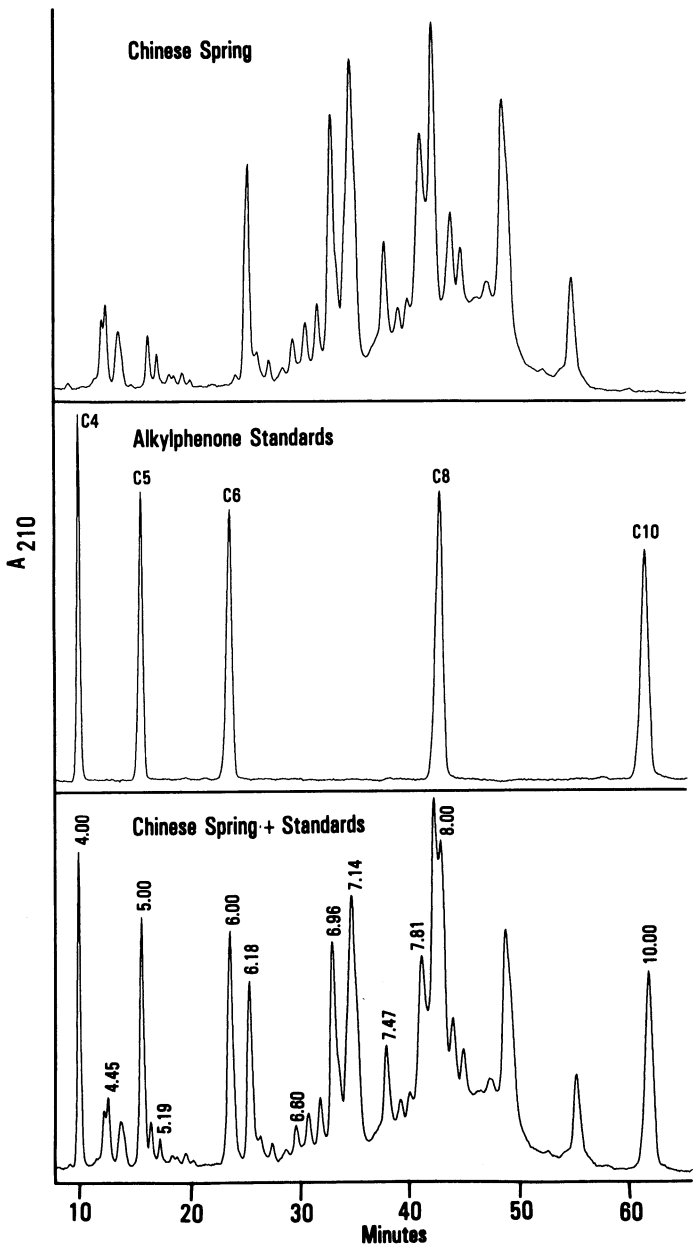

Fig. 3. RP-HPLC separations of Chinese Spring gliadin, a mixture of alkylphenone standards, and a mixture of these two samples on a SynChropak RP-P (C_{18}) column at 70°C, using a gradient from 25–50% CH_3CN (+ 0.1% TFA) during 55 min, with a total run time of 65 min.

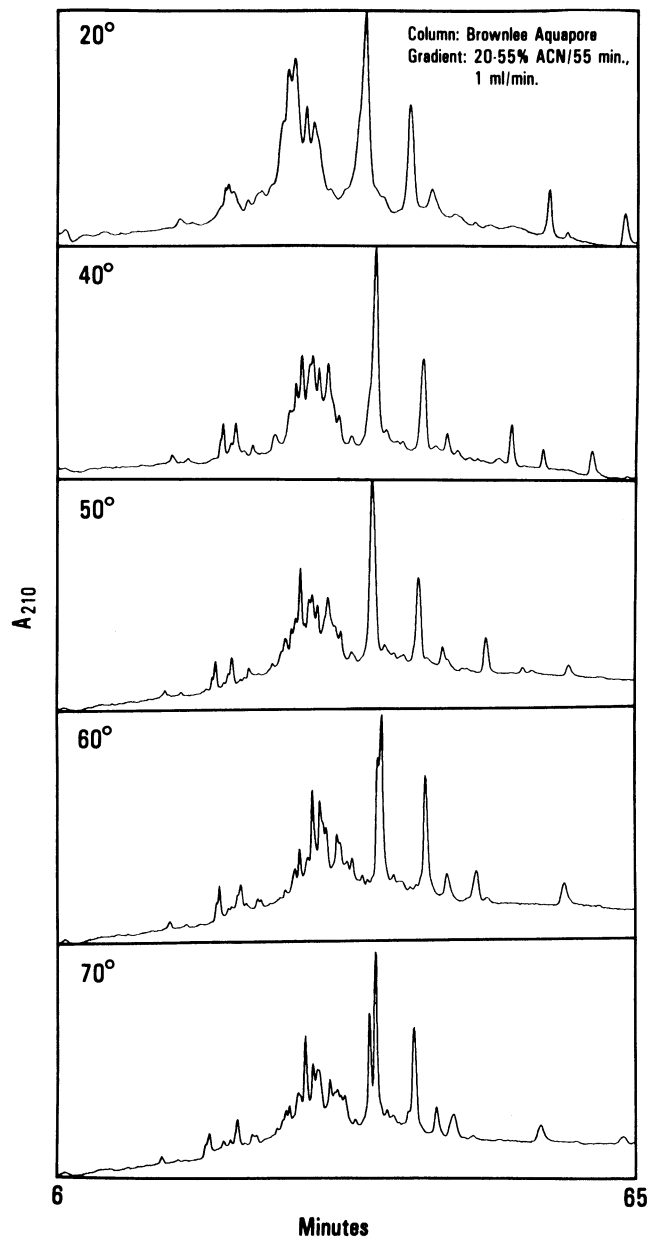

Fig. 4. RP-HPLC of a fresh Anza gliadin extract on a Brownlee Aquapore RP-300 column at representative temperatures from 20 to 70°C, using a gradient from 20–55% CH_3CN/55 min, with a total run time of 65 min, at a flow rate of 1.0 ml/min.

in varietal identification within and between laboratories. Kikta and Stange (1977) note that alkylphenones may react with primary amines above 50°C, suggesting potential problems with proteins; to date, however, we have noted no problems in analyzing gliadins with minimal exposure to standards. The suitability of standards for proteins must, however, be carefully considered before routine use.

Effects of Constant Temperature

In initial RP-HPLC studies of cereal proteins (Bietz 1983), a temperature of 30°C was generally used. Ambient temperature was also adequate for many purposes. When we more carefully investigated RP-HPLC elution time precision and reproducibility for Wanser gliadin peaks during replicate analyses at room temperature, however, we noted coefficients of variation higher than normal (0.28–0.96) (Table II, series 1 and 2). When these data were obtained, major temperature fluctuations were occurring within our laboratory; this seemed the likely cause of our results. At a constant temperature, however, of 70°C for 8 min (Arkan) or 55 min (Langdon) (Table II), or of 30°C for the same run times (results not shown), precision improved markedly, and average coefficients of variation were reduced to approximately 0.1 (as in Table I). These studies emphasize the absolute necessity of maintaining constant temperature during RP-HPLC when reproducibility is important.

The effects of temperature in RP-HPLC are becoming well known but are incompletely understood. RP-HPLC selectivity for low-molecular-weight solutes is influenced by temperature (Snyder 1979), and similar effects have been noted for large peptides and proteins (Mahoney and Hermodson 1980, Hearn 1980, Terabe et al 1981, Cohen et al 1984b). These effects may occur over a fairly narrow temperature range; generally, peptide or protein retention decreases as temperature increases.

Effects of Elevated Column Temperature

Most of our previous studies were done at 30°C or at room temperature; widely different temperature conditions were not examined. Indeed, there has been a concensus that, for proteins, RP-HPLC resolution frequently decreases at high temperature, perhaps because retention times decrease. In particular, the temperature range from 50–70°C has apparently not been thoroughly examined by others. We, therefore, re-examined the effect of temperature on RP-HPLC resolution of cereal proteins.

As noted in Figure 4, as temperature is increased, resolution markedly improves. For example, for the highest absorbance peak at about 31 min and for the major group of peaks at about 21–26 min, more peaks are resolved at higher temperatures, and peak sharpness improves. Much of the improved column performance occurs above 50°C; in general, resolution of gliadins, expressed as total number of discernible peaks and shoulders, is best at 70°C. Resolution at 60°C is slightly inferior to that at 70°C (compare, for example, the shoulder on the highest peak at 60°C with its resolution at 70°C), and resolution at 20–30°C is only about half that at 70°C. Similar effects have been noted with other RP-HPLC columns, and we now routinely use 70°C for protein separations. We have experienced no problems with decreased column lifetime under these conditions. We have not tried temperatures above 70°C, because they approach the boiling point of CH_3CN (82°C).

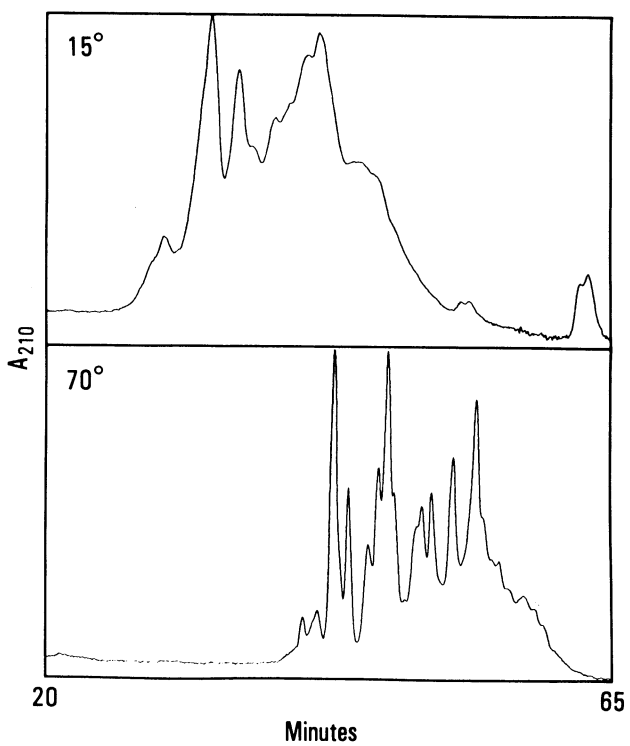

Fig. 5. RP-HPLC of 70% ethanol-soluble zein on a Brownlee Aquapore RP-300 column at 15 and 70°C. The sample, which had been dialyzed and lyophilized, was redissolved in 70% ethanol and separated using a gradient from 40–55% CH_3CN/55 min, with a run time of 65 min, at 1.0 ml/min.

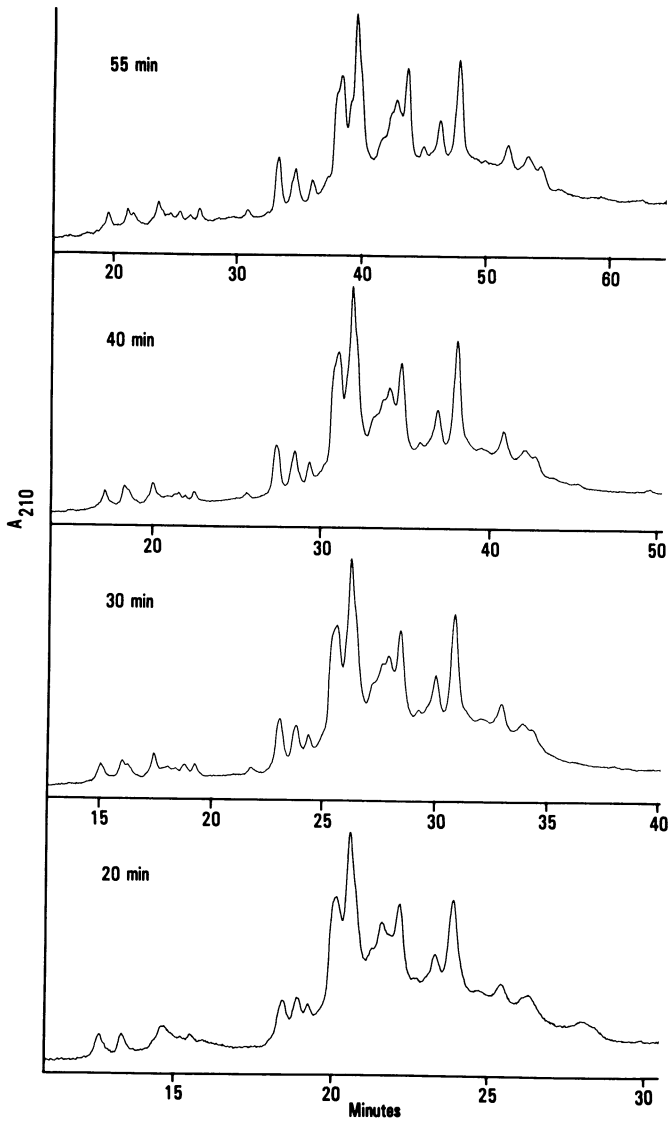

Fig. 6. Effect of gradient time on RP-HPLC resolution of Sage gliadin. Gliadin extract was chromatographed on a SynChropak RP-P (C_{18}) column at 70°C, using a gradient from 25–50% CH_3CN (+0.1% TFA) at 1.0 ml/min. Gradient times of 55, 40, 30, and 20 min were followed by isocratic elution at 50% CH_3CN for varying periods of time as indicated. Raw data were stored in analog form on a ModComp computer system, and subsequently replotted using a variable time scale to cause apparent coelution of corresponding peaks in the four chromatograms.

In part, the improved resolution noted at elevated column temperatures may represent altered column selectivity. Examination of Figure 4 reveals that elution times of various peaks may shift in relation to one another, emphasizing the importance of a constant and well-defined temperature in RP-HPLC analysis of gliadins, and demonstrating that improved resolution at higher temperatures is a complex phenomenon needing additional study.

Some reports (see above) indicate that RP-HPLC retention times for many peptides and proteins decrease with increased temperature. For gliadins (Fig. 4), some peaks also appear to shift to shorter elution times, while others remain relatively constant; no consistent trend can be noted for gliadins or other cereal proteins we have analyzed. Any existing trend is further complicated by the apparent change in column selectivity as a function of temperature.

RP-HPLC resolution of gliadins at 30°C (data not shown), as used before (Bietz et al 1984a,b), was essentially identical to that at 20°C (Fig. 4). In addition, we have found that RP-HPLC resolution of gliadins progressively decreases at temperatures below 20°C (data not shown); in contrast, Jones et al (1985) has observed improved resolution for purothionins at low temperatures, indicating that the magnitude and direction of temperature effects on resolution of cereal proteins vary widely with protein type.

The extent of improved resolution of cereal proteins at elevated temperature varies considerably with the sample. For fresh gliadin extracts (Fig. 4), association is probably minimal; nevertheless, marked improvement may occur. For older samples, which have been lyophilized and redissolved, the effect may be even greater. An extreme example, for zein (corn prolamin) extracted from inbred W64A with 70% ethanol, dialyzed, lyophilized, and subsequently redissolved in 70% ethanol, is shown in Figure 5. At 15°C (or at other temperatures near ambient), resolution was extremely poor. At 70°C, however, resolution was good, and most components eluted much later, similar to results from freshly extracted zein. Cereal endosperm storage proteins have marked tendencies to associate (Bekes et al 1983); this may be especially true for zein, one of the most hydrophobic proteins we have examined (Bietz 1983). It seems likely that zeins tenaciously aggregate upon dehydration, and upon rehydration resist dissociation to their initial state. Elevated temperature may disrupt noncovalent bonds between zeins or other cereal proteins, leading to complete disaggregation. Cole et al (1983) noted that, for gluten proteins, increasing temperature may dissociate hydrogen bonds; increased temperature generally promoted hydrophobic interactions, however.

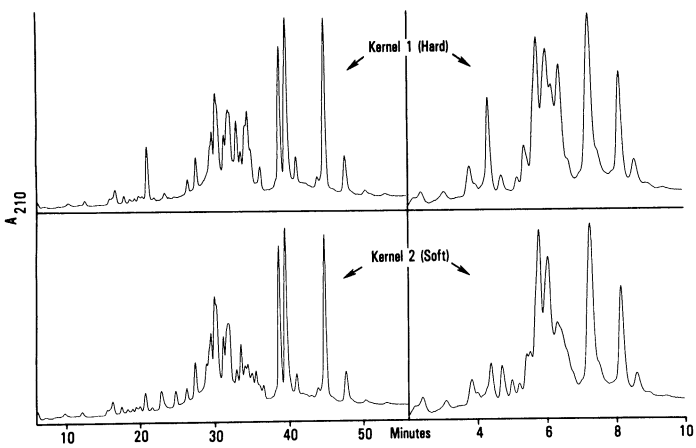

Fig. 7. Analysis of two phenotypically different (hard vs. soft) kernels, selected from a bulk sample of the hard red winter wheat variety Arkan, by 55 min and 8 min gradient RP–HPLC of extracted gliadin proteins. Samples were analyzed on a Brownlee Aquapore RP-300 (C_8) column at 70°C using a gradient of 25–50% CH_3CN during either a 55 min (at 1.0 ml/min with a total run time of 65 min) or 8 min (at 3.0 ml/min with a total run time of 10 min) gradient time.

Although increased temperatures might enhance column efficiency as a result of more rapid mass transfer (Hearn 1980), and, for certain proteins, might sharpen peaks or alter peak numbers due to changes in conformational states (including active and inactive forms, Cohen et al 1984a,b), for cereal proteins there seems to be a more complex explanation. Further studies are necessary to fully explain all effects, but the use of elevated (approximately 70°C) column temperature is highly recommended for cereal protein RP-HPLC.

Effect of Analysis Time

In our original RP-HPLC procedure for wheat varietal identification, only about 20 samples can be analyzed per day per instrument. We have now drastically decreased total analysis time

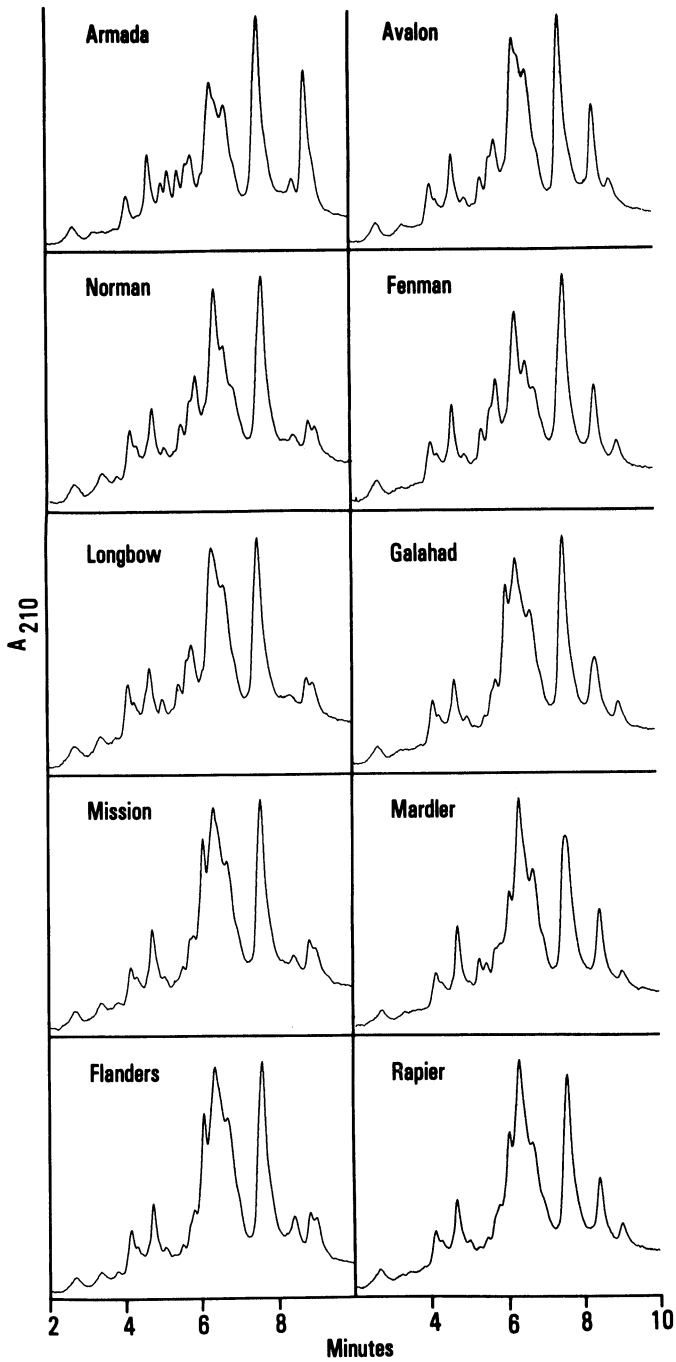

Fig. 8. Rapid RP-HPLC analysis of gliadins extracted from composite flour samples of ten English wheat varieties. All samples were analyzed on a Brownlee Aquapore RP-300 (C_8) column at 70°C using a gradient from 25–50% CH_3CN (+0.1% trifluoroacetic acid)/8 min, with a total run time of 10 min, an equilibration delay of 3 min, and a flow rate of 3 ml/min.

by using more rapid gradients and increased flow rates. Typical results for Sage gliadin, analyzed at 70° C and gradient times from 20 to 55 min, are shown in Figure 6.

Slight losses in resolution do occur as run time decreases, but similar results were obtained at about 60 and in 30 min. If those major features that permit differentiation can still be seen, analysis times faster than originally proposed may be advantageous (Bietz et al 1984a,b). Thus, the rate of sample analysis may be at least doubled.

Even more rapid analyses are possible. When two kernels were selected from the hard red winter wheat variety Arkan on the basis of having apparently "hard" or "soft" phenotypes, RP-HPLC under standard analyses conditions (Bietz et al 1984a,b) (chromatograms on the left, Fig. 7) revealed minor apparent differences. For example, kernel 1 has a major peak at about 21 min which is diminished in kernel 2, and kernel 1 also contains increased amounts of components at 33–37 min. Analysis of these same two samples at a gradient time of 8 min and flow rate of 3.0 ml/min results in the chromatograms on the right of Figure 7. Resolution is much decreased compared to standard analytical conditions: only about half as many peaks and shoulders are present. Nevertheless, the same major differences observed in long runs are still apparent, namely, amounts of components at around 4.3 and 6.4 min are greater in kernel 1 than in kernel 2. Thus, these apparently different genotypes are differentiated as easily in about 10 min as in 60 min. Including time for column re-equilibration (2–3 min) and sample injection (1 min), this procedure permits approximately 100 automated analyses per day per instrument. For many purposes of varietal identification, selection, or demonstration of homogeneity or heterogeneity, resolution by the rapid method is sufficient.

Examples of Wheat Varietal Identification Using Improved Procedures

When relatively few varieties are marketed in an area, such as England, and when each variety is homogeneous, fast RP-HPLC of flour samples (or single kernels) may readily differentiate all varieties, permitting identification and prediction of quality. For 10 major U.K. wheat varieties (Fig. 8), rapid RP-HPLC of flour samples revealed significant qualitative and quantitative differences. When such an analysis is combined with rapid protein extraction (Bietz et al 1984a) and sample clarification (filtration or centrifugation), it is now possible to identify a wheat, particularly from a relatively small set of varieties, in 15 min.

Selection from a population as well as detection of off-types or biotypes within a variety may also be achieved by rapid RP-HPLC. For example, there is an increasing trend toward genotypic variability in some newly introduced U.S. wheats, such as Newton, a hard red winter variety of excellent quality. To evaluate variability, 100 kernels of Newton were randomly selected and analyzed by rapid RP-HPLC; total analysis time was about one day, rather than the five days necessary with earlier procedures. The resulting chromatograms (Fig. 9) indicated considerable apparent heterogeneity for gliadins; nine of the many resulting pattern types, with their frequencies, are shown. Similar heterogeneity has been observed for other new varieties (results not shown). The significance of these observations is not yet totally clear; much more remains to be done. The results do suggest, however, the probability of more kernel-to-kernel variability at gliadin gene loci in new than in older varieties; breeders indicate that this reflects the different strategies now being used to produce high-quality wheats without breeding for genetic uniformity. RP-

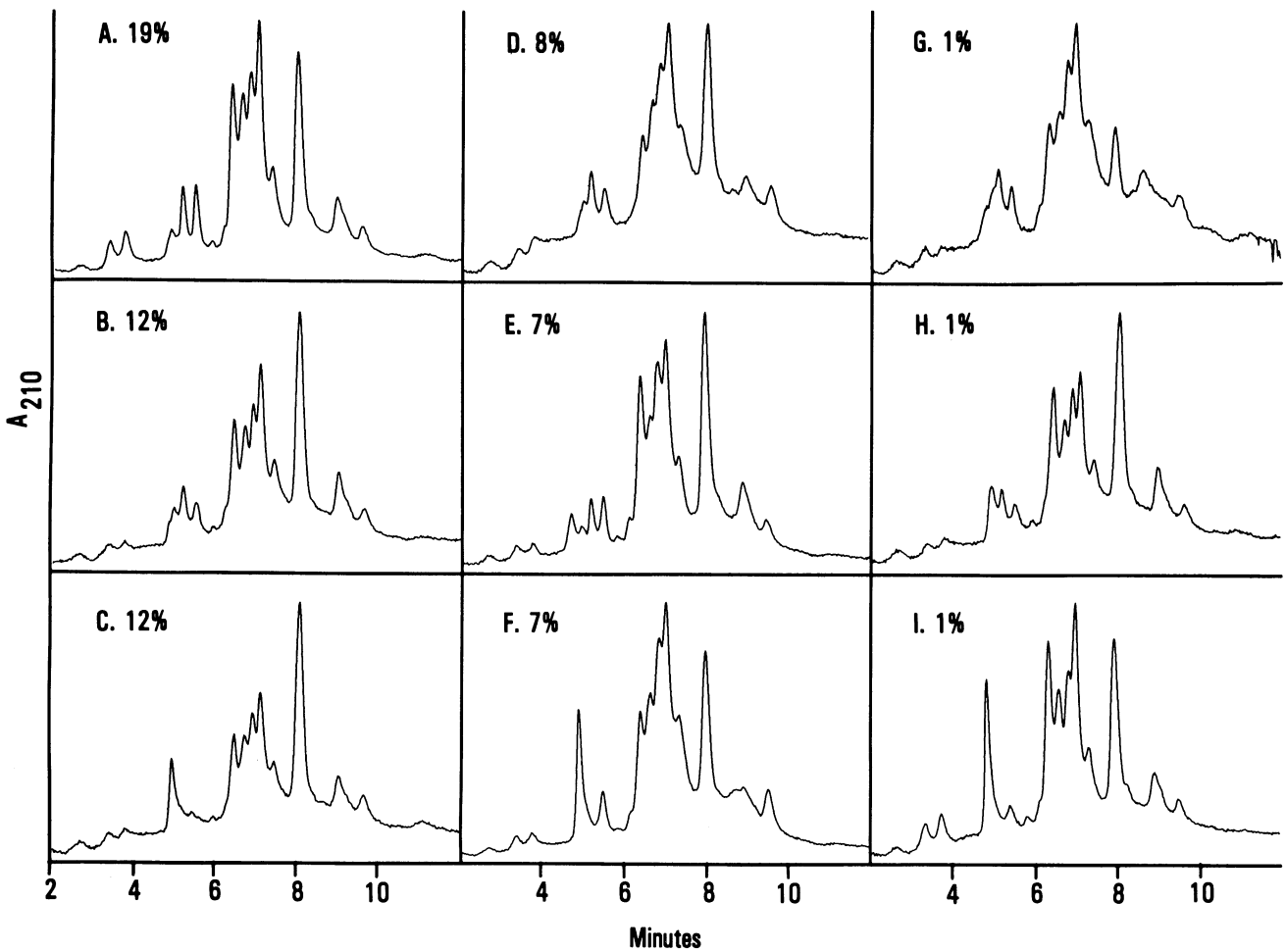

Fig. 9. Variability in the gliadin patterns of Newton hard red winter wheat as demonstrated by RP-HPLC. Samples extracted from single kernels were analyzed on a Brownlee Aquapore RP-300 column at 70° C and 2.5 ml/min, using a gradient from 25–50% CH_3CN/8 min, with a total run time of 10 min, and an equilibration delay of 3 min. Percentage figures indicate approximate frequencies of pattern types.

HPLC can be a valuable tool for breeders to analyze and detect any such variability.

In the United States, most wheat is marketed as mixtures of cultivars of the same class. Generally, identifying a sample by class, and determining protein content and possibly other characteristics, accurately predicts its quality and end-use potential. In some cases, however, it is still necessary to identify the varieties present. Analysis of sufficient individual kernels, as in Figure 9, is still too laborious for routine use; procedures for analysis of bulk samples are desirable. One potential method for analyzing mixtures is through computer analysis of gliadin RP-HPLC data (Fig. 10).

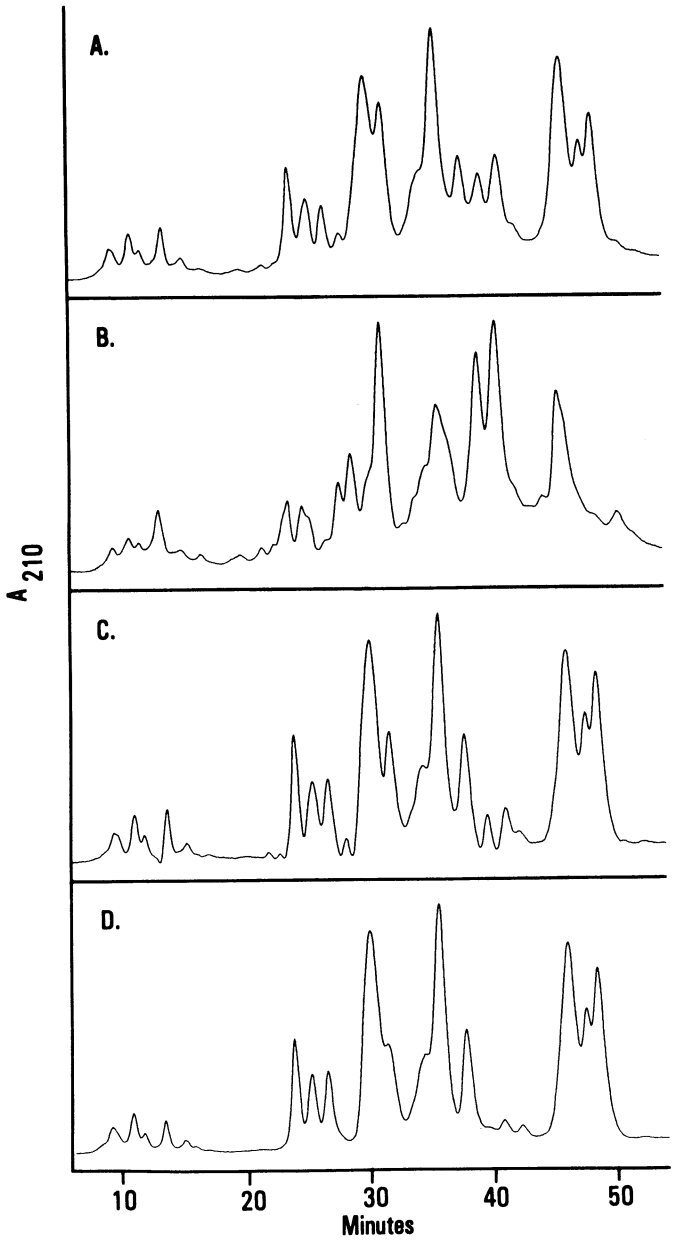

Fig. 10. Computer analysis of mixed wheat by RP-HPLC of extracted gliadins. Chromatograms of (**A**) 50% Centurk + 50% Langdon, (**B**) Centurk, and (**D**) Langdon were obtained on a SynChropak RP-P (C_{18}) column at 30°C using a gradient from 25–50% CH_3CN/55 min, with a total run time of 65 min, at a flow rate of 1.0 ml/min. The automatic difference curve (**C**) was obtained as described in the text. To facilitate comparisons, ordinates of all chromatograms were normalized so that, for each chromatogram, the highest peaks had equivalent heights. Consequently, absorbance scales for each chromatogram differ. Such comparisons are valid since overall "fingerprints" are characteristic of varieties (Bietz et al 1984b), but, within the limits of detector sensitivity and column capacity, are independent of sample size.

Varying mixtures were prepared from laboratory-milled flours of the wheat varieties Centurk (hard red winter) and Langdon (durum); an RP-HPLC chromatogram of gliadin from a 50/50 (w/w) mixture is shown in Figure 10A. Comparison of individual peaks in such a chromatogram to standard varieties should permit identification of one component of this binary mixture, Centurk (Fig. 10B). The computer program CHROCP, developed by R. O. Butterfield, was then used to automatically subtract the chromatogram of Centurk from that of the mixture. In this program, a difference curve is generated from voltage values at equivalent points on each chromatogram which minimizes negative baseline deflections; this is accomplished by optimizing the value of the constant K in the formula $A - KB = C$, where A = data set 1, B = data set 2, and C = the resultant difference curve. When CHROCP automatically subtracted the chromatogram of Centurk (Fig. 10B) from that of the mixture (Fig. 10A), a difference curve (Fig. 10C) was generated which was markedly similar to Langdon (Fig. 10D), permitting its identification.

In addition, by automatically determining K, the computer predicts amounts of each component. If equal amounts of samples are analyzed (giving approximately equal total integrated areas for the resulting chromatograms because of detection at 210 nm), K directly reveals the amount of B; in this case, the determined value, 0.4785, predicts about 48% Centurk (Fig. 10B) and 52% Langdon, close to the mixture's known composition. If unequal amounts of samples are analyzed, the computer-determined value of K must be divided by the ratio of the total integrated chromatographic area of the mixture to the area of the component being subtracted, giving an adjusted constant equivalent to that for equal amounts of sample, which then reveals quantities of each component. Since qualitative expression of gliadins varies little with environment or grain protein content, this quantitative approach should be generally valid. These results, though preliminary and subject to further verification, demonstrate the potential of computer analysis of RP-HPLC data to both identify and quantify components present in binary mixtures. In theory, this procedure should also apply to more complex mixtures.

CONCLUSION

Our results demonstrate improved reproducibility, resolution, and speed of RP-HPLC gliadin analysis for wheat varietal identification, achieved through the modification of solvent composition, gradient time, and temperature. Thus, we now routinely use H_2O + 1% TFA and CH_3CN + 0.1% TFA as solvents, rather than mixed H_2O/CH_3CN/TFA solvents (Bietz et al 1984a,b), to generate gradients with a two-pump system. We use a column temperature of 70°C, rather than 30°C or ambient conditions as before. And, for some applications, we use a gradient of about 8 min at 2.5–3.0 ml/min (with 2 min final hold and 3 min re-equilibration) rather than a 55 min gradient at 1.0 ml/min (with 10 min final hold and 10 min equilibration delay).

The qualitative and quantitative information in an RP-HPLC chromatogram is great and is frequently more than necessary. Thus, especially for routine or simple applications, it is sometimes desirable to decrease resolution and thereby maximize productivity. We have now defined alternative conditions for RP-HPLC analysis of wheat gliadin that significantly decrease analysis time by using rapid gradients and increased flow rates. Elevated column temperature (70°C) also improves resolution compared to analysis at ambient temperature. The overall result is that productivity may increase five- to sixfold, whereas resolution, measured by total number of peaks, decreases only about 50% compared to long analytical runs.

Altered solvent compositions, constant temperatures, and internal standards significantly improve reproducibility and data precision in RP-HPLC. Variability resulting from differences in solvent preparation or age, column age or type, changed flow rates, instrument performance, operators, or laboratories may be significantly reduced. Proteins may be identified in terms of a "hydrophobicity index" determined by comparing their elution times to standards, thus normalizing elution characteristics and

facilitating data comparisons by eliminating much experimental variability. Because of these improved procedures, computer examination of RP-HPLC data may now be a feasible way to automatically identify varieties, with no subjective evaluation, by integration, normalization, and comparison of data to a library of standards.

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