Statistical Analyses of Gliadin Reversed-Phase High-Performance Liquid Chromatography Patterns of Hard Red Spring and Hard Red Winter Wheat Cultivars Grown in a Common Environment: Classification Indices

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ABSTRACT

Reversed-phase high-performance liquid chromatography was used to analyze gliadins extracted from grain harvested from 12 hard red winter (HRW) and 12 hard red spring (HRS) wheat cultivars grown in a common environment. Visual examination of the gliadin patterns did not distinguish the two wheat classes by the presence or absence of any one particular peak. The peak heights at each time interval from each cultivar were analyzed through cluster, principal component, and canonical analyses. Cluster analyses, based on closest (Euclidean) distances, produced five clusters plus six HRW cultivars that did not fall into any cluster. In the five clusters, two contained only HRS cultivars, two contained only HRW cultivars, and one contained both HRW and HRS cultivars. Principal component analysis showed that the first principal component (PC1) explained 21% of the total variation among cultivars, primarily separating HRW and HRS classes with only minor overlap. The first three principal components together explained nearly half (44%) of the total variation. In these three major dimensions, there was greater scatter within the HRW class than within the HRS class. Canonical analyses demonstrated that the correlation between PC1 and the first canonical variable was 0.79, indicating that the cultivars and classes were in similar groups. Even though both PC1 and the first canonical variable separated HRW and HRS classes, HRW cultivars occurred among the HRS cultivars in both analyses. Canonical discriminate analysis, based on gliadin reversed-phase high-performance liquid chromatography, allocated all cultivars to their correct classes, except for the HRW cultivars TAM 105 and TAM 107.

Cereal cultivars can be readily identified by reversed-phase high-performance liquid chromatography (RP-HPLC) of their storage proteins (Burnouf et al 1983, Bietz et al 1984, Bietz and Cobb 1985, Lookhart 1985a, Lookhart et al 1987, Scanlon et al 1989). The RP-HPLC method has been shown to be fast, accurate, reproducible, and relatively easy to perform; furthermore, the data are readily analyzed and quantitated.

The relationship of certain cereal proteins to quality has been the subject of considerable study for many years. Finney (1943) was among the first to show the relationship of wheat flour proteins to bread-making quality by fractionation-reconstitution work. Gliadin storage proteins in wheat are encoded at six loci on the group 1 and 6 chromosomes (Boyd et al 1967). Damideaux et al (1980) used polyacrylamide gel electrophoresis and Autran et al (1988) used HPLC to show the relationship of durum wheat quality to specific gliadin proteins, designated gamma 42 and gamma 45. Huebner and Bietz (1987) reported the presence of a peak for predicting quality in gliadin HPLC patterns of spring wheats.

Various computer-oriented techniques have been used to analyze RP-HPLC data; the most common included pattern recognition and statistical analysis. Qualitative analyses of electrophoregrams, and chromatograms using pattern recognition for varietal identification and other classification purposes, have been described by Lookhart et al (1983), Cohen et al (1987), and Scanlon et al (1989). Huebner and Bietz (1987) reported that computer analysis of RP-HPLC data may help identify proteins related to bread-baking quality. In an effort to determine the cultivar composition in mixtures, Marchylo et al (1988) used computer analysis of RP-HPLC data and concluded that further improvements in methods were necessary. Endo et al (1990a,b) used statistical analysis on RP-HPLC data from blends of hard red spring (HRS) and hard red winter (HRW) wheats and attempted to relate the HPLC data to milling data. Little difference was found for the elution times of the main peaks of the HRS or HRW blends. Since the RP-HPLC patterns of the HRS blends analyzed by Endo et al (1990a) were nearly identical, the mixture of varieties must have been very similar. This effect was also noted for the HRW blends. However, the pattern for the HRS blends were different from the pattern of the HRW blends, although many spring and winter wheats exhibit similar gliadin electrophoretic patterns (Jones et al 1982, Lookhart 1988).

Classification of wheat as winter or spring in the U.S. is historically based on the season of the year that the seeds are planted. Winter wheats are seeded in the fall, whereas spring wheats are seeded in the spring. Perceived differences in baking or milling quality between these classes have generated considerable effort towards differentiating them. However, large environmental variations are primarily responsible for differences in quality, both within and among cultivars (Peterson et al 1992).

In this study, we statistically analyzed the RP-HPLC data of gliadin protein patterns of 12 individual cultivars of HRS and 12 of HRW. All wheats were sown and harvested at the same location (California) and at the same time in 1988 to reduce the influence of environmental conditions on the properties of each cultivar. Environment, however, has only a minor effect on gliadin patterns (Lookhart et al 1984, Lookhart 1985b).

MATERIALS AND METHODS

Samples

Grain from 12 HRW and 12 HRS wheat cultivars (Table I), grown in a randomized block design with two replicates, during the same season and at the same location near Sacramento, CA, were obtained from Rob Bruns, Agripro Biosciences Inc., Berthoud, CO.

HPLC

The HPLC procedure was that of Lookhart et al (1986), with the exceptions noted below. Ground wheat or flour samples (250 mg) were extracted with 0.750 ml of 70% ethanol for 30 min at room temperature and centrifuged at 4,000 × *g* for 10 min at 25° C (Beckman J-6B). The supernatant was decanted and stored at room temperature for up to four days until analysis by RP-HPLC. Extracts (10 μl) were analyzed in duplicate on a Hewlett-Packard 1090 Chromatography Data system by elution from a Vydac RP (C18) column (218TP54) at 1 ml/min. The elution conditions consisted of a multistep linear gradient; the starting solvent contained 25% acetonitrile and 75% water, each containing 0.1% trifluoroacetic acid. The acetonitrile concentration increased to 35% at 5 min, 50% at 10 min, 75% at 17 min, 85% at 18

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min, and then returned to the initial conditions (25% acetonitrile) at 19 min. The total runtime, from injection to injection, was 30 min, which included an 11-min reequilibration step between runs. A Hewlett-Packard 1040A diode-array detector was used for detecting the eluted components at 210 nm (0.500 absorbance units full scale). A data point was stored every 640 msec on a Hewlett-Packard (9000-300) computer for subsequent integration, replotting, and comparison.

Statistical Analysis

For each sample, the presence or absence of a peak was determined by Hewlett-Packard peak-detection software for each 0.1-min chromatographic time interval. Over all samples, there were 83 such time intervals between 3.3 (void volume peak) and 14.0 min in which peaks were found. In the 25 time segments (108–83) where no peaks were found, there was no information for cultivar comparison. After elimination of peaks occurring in three or fewer samples, these time intervals were represented by 59 variables for each sample. The value of each variable was either the baseline-corrected absorbance (peak height, if there was a peak at that elution time) or 0 (if there was not). Most cultivars exhibited 25–30 peaks, however, so there were between 29 and 34 time intervals for each cultivar where no peaks were found, but they were utilized in the analyses.

The number of cultivars of each class with a value greater than zero was computed for each elution-time variable. The mean of each variable (peak height) over the duplicate injections of each of the two replicates of each cultivar, divided by the sum of all peak heights for the respective sample, was computed and used to perform cluster analysis, principal component analysis, and canonical discriminant analysis (Karson 1982) using statistical analysis software (SAS 1988).

In cluster analysis, the peak heights of the 59 retention times most common among the 24 cultivars were used to compute a Euclidean distance between every pair of cultivars. The unweighted-pair-group method, based on arithmetic averages, was used; the Euclidean distances were not squared (METHOD = AVERAGE NOSQUARE in SAS). Clustering finds the shortest distances between any two cultivars, groups them, then calculates next closest distance between cultivars or groups, and continues until all cultivars and groups are joined. Distances for grouping are subjectively assigned to provide a reasonable number of groups with some physical significance.

Principal component analysis utilized the same 59 variables for each of the 24 cultivars as for the cluster analysis. Based on the correlation matrix, 59 linear combinations of the 59 variables (i.e., principal components) are calculated for each cultivar, such that no correlation exists between any two principal components, and principal components are ordered by the variance they account for. Thus, the first component accounts for

TABLE I
Cultivars Analyzed by Reversed-Phase High-Performance Liquid Chromatography (HPLC) and Their Pedigrees

Sample No.	Cultivar	Wheat Protein Content[a] (%)	Pedigree
Hard Red Winter			
1	Arkan	13.8	Sage/Arthur
2	Brule	12.5	(Ponca/3*Cheyenne//Selkirk/2*Cheyenne, NE68723)/7/Seu Seun 27/3/(CI12500, Nebraska 60//Mediteranean/Hope)/4/Pawnee/Cheyenne/5/Cheyenne/Ponca//Turkey Red/Cheyenne, NE68719)/6/Gage
3	Centurk 78	13.5	Kenya 58//Newthatch/3/Hope/2*Turkey Red/4/Cheyenne/5/Parker (Centurk Sel.)
4	Eagle	13.8	Scout Sel.
5	Hawk	12.8	(Sonora 64A//Tezanos Pintos Precos/Nainari 60, 11-18889)/3/Trapper/4/CO652643/5/Baca
6	Newton	13.1	Pitic 62/5/(Kenya58/Newthatch//Thatcher/3/Frontana/Thatcher, 11-53-526)/4/Sonora 64/6/Sonora 64/Klein Rendidor/7/Scout
7	Siouxland	13.6	Warrior*5/Agent//Kavkaz/3/Warrior*5/Agent
8	Scout 66	14.0	(Nebred//Hope/Turkey Red/3/Cheyenne/Ponca) Scout Sel.
9	TAM 101	13.5	Norin 10/3/Nebraska 60//Mediteranean/Hope/4/Bison
10	TAM 105	12.5	"Short Wheat"/Sturdy Bulk Sel.
11	TAM 107	12.0	TAM 105*4/Amigo
12	Vona	12.5	11-21183/6/(CO652363, Warrior/4/Kenya 58/Newthatch/3/2*Cheyenne/Tenmarq//Mediteranean/Hope/5/Parker)/7/Lancer/KS62136
Hard Red Spring			
13	Butte 86	14.2	Butte*2/3/ND 551//Butte*2/ND507
14	Coteau	14.9	ND496 sib//ND487/Fletcher
15	Era	12.7	(MN2824*2/11-50-72, 11-55-10, CI15523)/4/(11-53-546, CI13405, Pembina/11-52-329/3/11-53-38/111-58-4//11-44-29/3*Lee)
16	Guard	13.5	Eureka/Dawn
17	Len	14.3	ND499/3/Justin/RL4205//Wis261
18	Marshall	13.4	Era/Waldron
19	Nordic	12.7	Web 13-3/MN7125
20	Pioneer 2369	13.2	Era/3/Tezanos Pintos Precos/Sonora 64//Chris
21	Stoa	14.4	ND527/3/ND496 sib.//ND487/Fletcher/4/Era
22	Telemark	13.9	Marshall/Len
23	Waldron	15.3	Lee/Mida/Kenya 338AA/3/Lee/4/Justin
24	Wheaton	13.3	Crim/2*Era//Buitre/Gallo

[a] Protein content listed on a 14% moisture basis.

the largest amount of variation among cultivars. The second principal component explains the second largest amount of variation, and so on. Each variable is assured equal weighting in principal component analysis by the use of the correlation matrix instead of the covariance matrix.

For canonical analysis, we used the variables from the first principal component with the seven largest coefficients. This was necessary because the number of variables must be smaller than the number of objects (cultivars) in canonical analysis. Additionally, beyond the seventh coefficient, there was a gap in their size and they diminished rapidly. The first canonical variable provides maximum separation of known classes—in this case, winter and spring wheats. We used canonical discriminant analysis to remove one cultivar at a time, classify it on the basis of its canonical variable values, and determine the probability of the cultivar being classified correctly.

RESULTS AND DISCUSSION

The wheat protein contents and pedigrees for the 12 HRW and 12 HRS cultivars in this study are given in Table I. The RP-HPLC patterns of the 12 HRW cultivars are shown in Figure 1; those of the 12 HRS cultivars are shown in Figure 2. Essentially identical RP-HPLC patterns were found for the replicated analyses of duplicate samples (pattern overlays were identical). Therefore, the peak retention times and peak heights for a given cultivar were reproducible over the period of analyses. Over an extended period (six months to one year of column use), peak retention times would vary, but normally, the peaks would still be within the peak window of 0.1 min. Since the relative percent peak height was used in the analyses, small variations in peak heights would not affect the results. Visual examination of the patterns of the HRW and HRS cultivars allowed differentiation

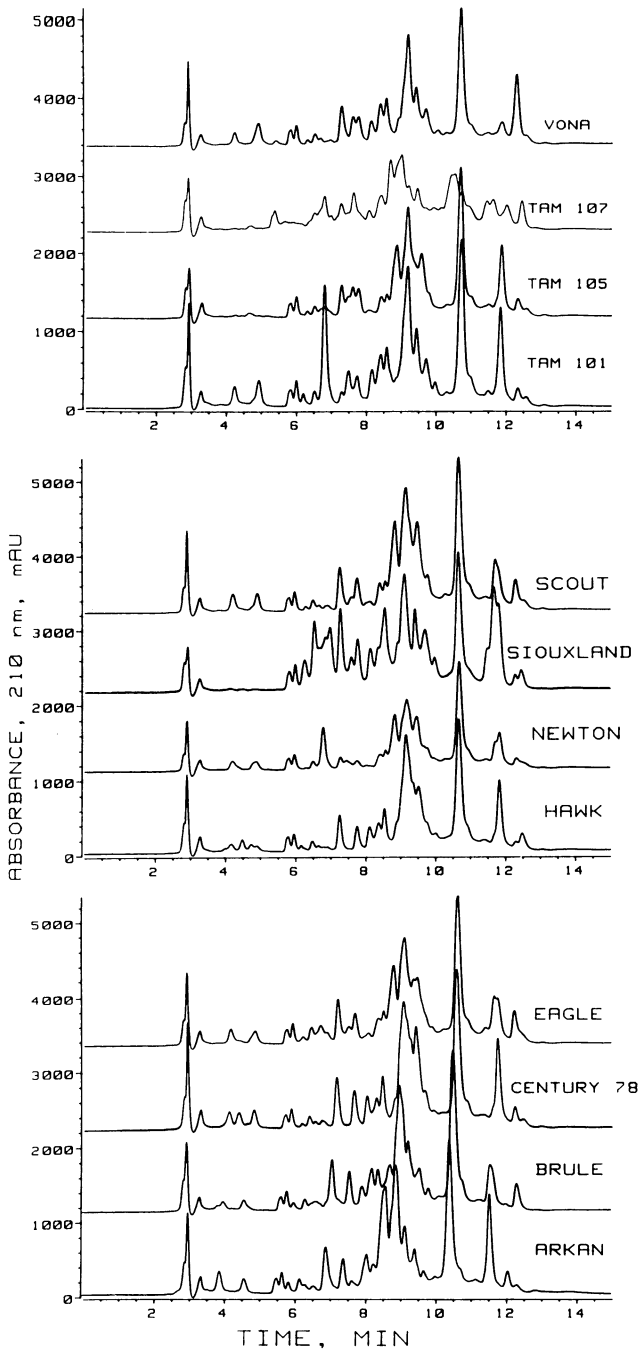

Fig. 1. Reversed-phase high-performance liquid chromatography patterns of gliadins (70% ethanol-soluble proteins) extracted from 12 hard red winter wheat cultivars. Extracts (10 μl) were analyzed on a Vydac C18 column by elution with a water-acetonitrile gradient at 45°C.

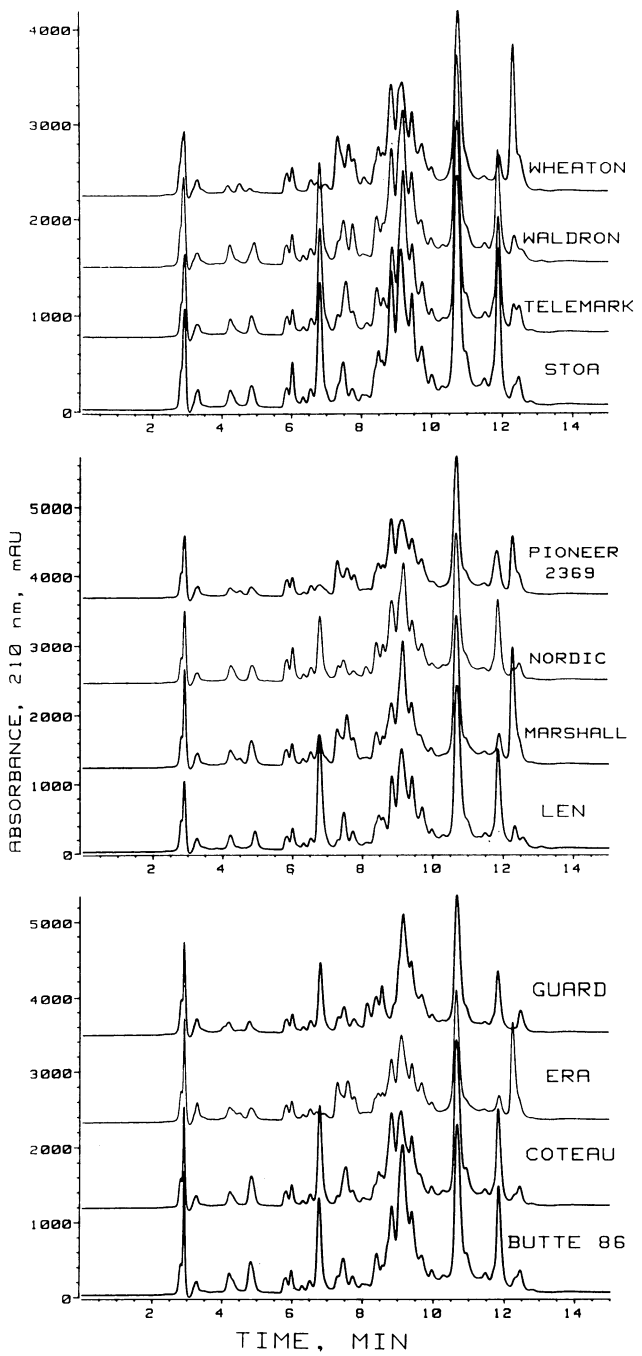

Fig. 2. Reversed-phase high-performance liquid chromatography patterns of gliadins (70% ethanol-soluble proteins) extracted from the 12 hard red spring wheat cultivars. Extracts (10 μl) were analyzed on a Vydac C18 column by elution with a water-acetonitrile gradient at 45°C.

of each variety but did not allow segregation of class. In other words, by visual analysis, no peak or group of peaks was characteristic of any class. Therefore, the peak elution times and heights were analyzed by statistical methods.

In the cluster analysis, the first branch point (d = 1.20) of the dendrogram (Fig. 3) separated four dissimilar HRW cultivars (Arkan, Brule, Centurk 78, and Eagle, all in Fig. 1) from all others. Those cultivars were very dissimilar from each other and from all other groups of cultivars. Their pedigrees (Table I) showed no similar backgrounds. When the maximum within-group distance was set at 0.75, five clusters were produced: the four cultivars listed above, plus two other HRW cultivars (Vona and Siouxland) that laid outside any cluster. None of their pedigrees appeared closely related.

Of the five clusters, two (D and E) contained all HRS cultivars, and two (A and B) contained all HRW cultivars. The fifth cluster (C) contained five HRS and one HRW cultivar (TAM 101). Cluster B, comprising TAM 105 and its backcross derivative, TAM 107, was more similar to the predominantly HRS clusters than to the HRW ones. Looking at their pedigrees, there is no obvious genotypic explanation for this, but nothing is known about the cultivar listed as "short wheat" in their pedigrees (Table I).

The pedigrees for each of the two clusters of HRS cultivars were similar. The all-HRS cluster D contained Era, Wheaton, Pioneer, and Marshall, all of which had pedigrees of Era or Era backcrosses. In the HRS cluster E (Coteau, Len, and Stoa cultivars), Coteau and Stoa had similar pedigrees, whereas Len contained North Dakota breeding material but not the same line number. The HRW cluster A (Hawk, Newton, and Scout 66) contained cultivars with some similarities in pedigrees, including Scout and Sonora 64 parents. In the HRW cluster B, TAM 107 is a backcross derivative of TAM 105.

The proportions of total variation accounted for by the eigenvalues of the first three principal components (Table II) were 0.21, 0.12, and 0.11. Therefore, these three principal components accounted for 44% of the variation in all 59 dimensions (peak retention times and heights). This indicated considerable correlation structure in the data set. If all the variables were independent, the three components would have accounted for only 5.1% of the total variation.

In a plot of these three components (Fig. 4), there was much greater dispersion among HRW than among HRS cultivars. The HRS cultivars formed two very restricted groups. One group comprised three cultivars (Era, Marshall, and Wheaton) released by the Minnesota-USDA program, plus Pioneer 2369 (the same grouping found by cluster analysis). The other group primarily

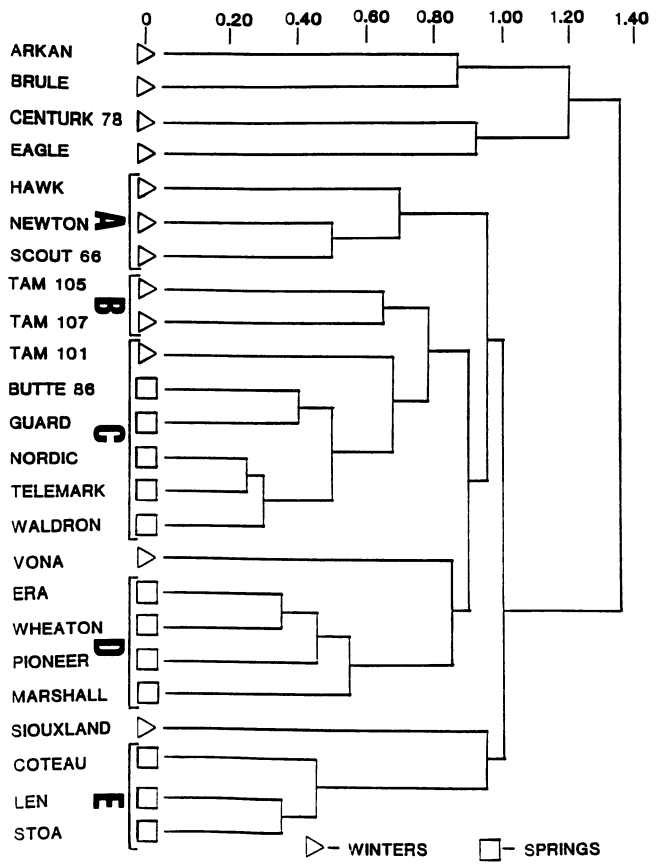

Fig. 3. Dendrogram of cluster analysis of the combined 12 hard red winter (△) and 12 hard red spring (□) wheat cultivars. The first branch point (d = 1.20) separated four dissimilar winter cultivars (Arkan, Brule, Centurk 78, Eagle) from all others. Clusters **A** and **B** contain all winter cultivars; **C** contained five spring and one winter cultivar (TAM 101); **D** and **E** contained all spring cultivars. Two winter cultivars (Vona and Siouxland) laid outside any cluster.

TABLE II
First Three Principal Component (PC) Coefficients for 20 Peak Time Variables with at Least One Coefficient with an Absolute Value of 0.20 or Greater (from a Total of 59 Peak Times Analyzed) and Eigenvalues for the Three Components

Peak Time (min)	PC1	PC2	PC3
6.0	−0.23	−0.09	0.03
6.7	0.07	−0.09	0.25
6.8	−0.20	0.11	−0.14
7.2	0.15	0.25	0.15
7.3	−0.04	−0.26	0.22
7.7	0.05	0.32	0.03
7.8	−0.02	−0.24	0.25
8.2	0.16	−0.07	−0.22
8.3	0.20	0.13	−0.06
8.5	0.03	0.11	0.21
9.0	0.19	−0.12	−0.25
9.1	0.11	0.20	0.05
9.5	0.10	0.01	0.20
10.2	0.21	0.18	0.03
10.6	0.20	0.22	0.05
10.7	−0.24	−0.06	0.11
11.5	−0.21	−0.07	−0.07
11.6	0.16	−0.10	−0.25
11.7	0.06	−0.09	0.21
11.9	−0.21	0.12	−0.15
Eigenvalue	12.4	7.5	6.6
Percent	21	12	11

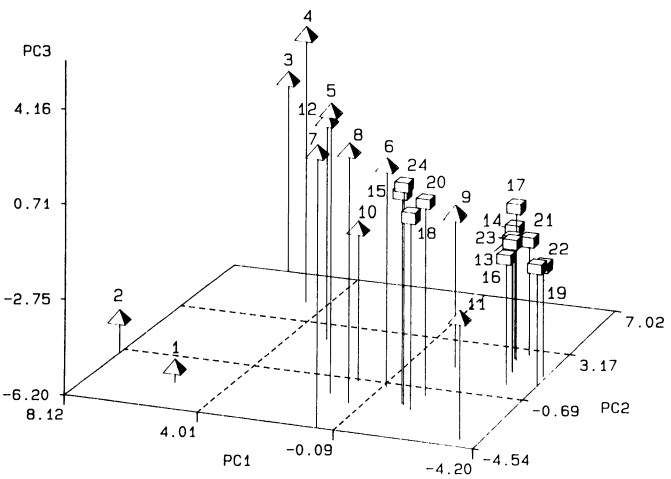

Fig. 4. Plot of first three canonical variables (principal component PC1, PC2, and PC3) showing separation of each cultivar and class in each dimension. △ = hard red winter wheat cultivars, □ = hard red spring wheat cultivars. **1-24** = Arkan, Brule, Centurk 78, Eagle, Hawk, Newton, Siouxland, Scout, TAM 101, TAM 105, TAM 107, Vona, Butte 86, Coteau, Era, Guard, Len, Marshall, Nordic, Pioneer 2369, Stoa, Telemark, Waldron, and Wheaton, respectively.

contained cultivars released in North Dakota. HRW and HRS cultivars were separated in Fig. 4, with the exception of Era, Marshall, Pioneer 2369, and Wheaton, which formed a close-knit group apart from the other HRS cultivars. Newton, TAM 101, and TAM 105 were close to that HRS group (Fig. 4). Arkan and Brule were within the broad HRW grouping but not close to any other cultivars.

With only two classes, HRW and HRS, consisting of 24 cultivars used for canonical analysis, it was necessary to limit the number of variables. Therefore, only the seven peaks (elution times of 6.0, 6.8, 8.3, 10.2, 10.7, 11.5, and 11.9 min) that had the largest coefficients in the first principal component were used to determine the first canonical variable. The squared canonical correlation for this variable was 0.74, and its simple correlation with the first principal component (computed from the same seven variables) was 0.79. Therefore, the axis giving maximum separation of the classes was oriented similarly to the primary axis for the total distribution of the cultivars. In other words, the cultivars are grouped similar to their class.

Canonical discriminant analysis was performed 24 times, omitting each cultivar in turn and then classifying that cultivar, based on the discriminant function. All cultivars were reclassified correctly, with the exception of TAM 105, which showed a 0.96 posterior probability of being classified with HRS cultivars. There was some ambiguity in the classification of TAM 107, which had a 0.39 posterior probability of falling into the HRS class. These two cultivars were also described as similar to HRS by cluster analysis. TAM 105 was found very close to the Era, Marshall, Wheaton, and Pioneer group by principal component analysis (Fig. 4). The mean probability of misclassification for HRW cultivars, other than TAM 105 or TAM 107, was 0.010; for HRS cultivars, it was 0.038.

CONCLUSIONS

Although 59 variables were analyzed, they represented only six genetic loci. Therefore, those analyses reflect only similarities in gliadin proteins, not overall relationships among cultivars. Principal component and canonical analyses took into account correlations of linked peaks, whereas the cluster analysis did not. The reason TAM 101 was misclassified in principal component analysis and TAM 105 was misclassified in canonical analysis is unknown. It may be because we used 59 variables in the former analysis and only seven in the latter. Since principal component analysis was based on all 59 variables, it separated classes at least as well as canonical analysis. Principal component analysis, therefore, probably provided the most complete picture of gliadin HPLC pattern variation among the HRW and HRS cultivars in this study. Classification of current cultivars may be possible with a system similar to this, but continued winter-spring crosses would eliminate its effectiveness.

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