Differentiation of Wheat Based on Fluorescence, Hardness, and Protein

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ABSTRACT

Fluorescence intensity, hardness, and protein data on whole wheat flours were analyzed using a discriminant function to classify wheats. Six classes and subclasses of wheat were represented in a set of 77 samples. When red wheats and durum were considered, a high correlation ($r = -0.907$) between hardness and fluorescence was obtained; however, when white wheats were included, the correlation dropped ($r = -0.05$). Discriminant analysis allowed separation of wheat into proper classes for 100% of durum, hard red spring, club, soft red winter, and soft white winter wheats; and for 94% of hard red winter wheat.

Factors most important in categorizing wheats for utilization include kernel hardness, size, and color, protein content and protein strength (Williams et al 1988). Kernel hardness (endosperm texture) was shown to be an important parameter in defining Canadian wheat quality (Fowler and de la Roche 1975) and is often used in wheat classification (Obuchowski and Bushuk 1980).

Several methods have been developed to measure wheat hardness characteristics including various grinding procedures and near-infrared reflectance spectroscopy (NIR) (AACC 1983, Anderson et al 1966, Gaines et al 1987, Miller et al 1984, Osborne 1984). Single-kernel measurements have shown that variability may be attributed to differences within a given class or variety (Pomeranz et al 1988a). Several methods have been shown to be highly correlated (Williams 1979, Miller et al 1984), suggesting that the simpler, faster methods may be used reliably.

Numerous factors contribute to wheat classification; therefore, one measurement such as hardness may not allow absolute discrimination of classes. Combining several variables may provide better classification, limiting the amount of overlap that occurs with a single variable. Measurements that add another dimension, not necessarily correlating with existing measurements, may be useful as wheat classification variables. Fluorescence may be such a variable.

Intrinsic or autofluorescence characteristics of wheat have been suggested for quantification of botanical components in wheat mill streams (Jensen et al 1982). Autofluorescence of wheat may vary between classes, and perhaps these differences may be added to classification schemes available and decrease the amount of overlap between classes. Certain of these characteristics are presented here and compared with hardness and protein data. This work represents part of a cooperative study between the U.S. Department of Agriculture (Federal Grain Inspection Service and Agricultural Research Service) and several state agricultural experiment stations as described by Pomeranz et al (1988b).

MATERIALS AND METHODS

Wheat Samples

Seventy-seven wheat samples were selected by the Federal Grain Inspection Service and the Agricultural Research Service to cover a range of hardness values and sent to the Western Regional Research Center (WRRC) for analysis. The 77 samples included 11 durum (DUR), 16 hard red winter (HRW), 10 hard red spring (HRS), 10 soft red winter (SRW), 20 soft white winter (SWW), and 10 club (CLB). Forty-one samples (Table I) were nursery stock, pure varieties from plant breeders, and 36 samples were from marketing centers (Table II, detailed descriptions in Pomeranz et al 1988b). All samples were pooled for this study.

TABLE I
Data for Nursery Stock Samples

Class and Cultivar	Fluorescence Intensity[a]				Hardness	Protein (as-is)
	P_{470}	A_{470}	P_{365}	A_{365}		
Durum						
Westbred	36.9	0.441	56.3	0.255	111.4	13.8
Vic	34.9	0.469	53.0	0.283	108.2	13.5
Mexicali 75	40.5	0.402	61.5	0.217	104.8	12.2
Cando	38.9	0.423	55.3	0.264	118.2	15.1
Monroe	32.9	0.493	51.4	0.298	111.0	14.2
Aloura	40.4	0.402	62.6	0.209	119.4	10.7
Hard red spring						
Oslo	25.2	0.621	38.3	0.420	44.5	14.5
Yecora Rojo	29.9	0.542	50.4	0.308	83.3	13.7
Stoa	29.4	0.555	45.2	0.355	85.3	14.8
Marshall	28.0	0.575	40.8	0.400	77.4	13.5
Len	24.0	0.635	38.6	0.424	81.9	14.9
Hard red winter						
Bennett	31.8	0.518	46.1	0.347	84.9	13.9
Newton	27.8	0.578	44.0	0.372	58.6	12.0
Lindon	26.7	0.601	42.9	0.382	56.5	11.0
Colt	25.6	0.616	41.1	0.397	59.5	10.8
Lancer	30.9	0.526	43.0	0.378	76.5	14.3
Probrand 830	27.0	0.589	43.7	0.371	71.4	12.4
Hawk	27.6	0.583	44.1	0.366	72.0	11.9
Bounty 203	25.8	0.617	44.0	0.366	48.7	12.9
TAM105	26.2	0.603	42.7	0.381	67.6	11.4
TAM105	28.8	0.560	43.1	0.377	66.6	12.2
Soft red winter						
Adena	20.7	0.706	42.4	0.387	21.0	10.4
Caldwell	23.0	0.661	46.1	0.345	29.8	9.8
Titan	23.2	0.660	44.4	0.366	27.3	9.7
Caldwell	20.9	0.698	43.0	0.380	27.7	9.8
Hillsdale	25.3	0.620	43.9	0.370	33.6	13.5
Soft white winter						
Daws	33.6	0.492	56.2	0.261	20.9	8.6
Frankenmuth	31.3	0.522	54.8	0.271	29.8	10.4
Stephens	35.8	0.468	57.7	0.249	27.3	9.3
Nugaines	32.0	0.512	57.9	0.250	24.2	8.8
Houser	38.9	0.424	55.4	0.267	20.9	10.5
Hill	29.3	0.554	49.6	0.317	34.5	11.9
Augusta	37.8	0.442	54.7	0.270	34.0	10.8
Hill 81	32.7	0.508	48.6	0.323	27.2	12.2
Purcell	39.2	0.427	56.1	0.262	21.1	11.1
Tecumseh	39.5	0.423	49.5	0.315	30.7	11.7
Club						
Crew	44.2	0.375	53.8	0.278	30.0	12.4
Moro	46.8	0.343	53.8	0.281	22.1	11.5
Tres	39.1	0.429	53.9	0.278	31.6	10.1
Tyee	51.2	0.313	54.3	0.275	31.4	12.5
Crew	40.3	0.414	53.2	0.283	34.0	10.4

[a] P_{470}, A_{470}; Relative fluorescence intensity (percent and absorbance) using filter cube 470 (450-490 nm excitation). P_{365}, A_{365}; Relative fluorescence intensity using filter cube 365 (365 nm excitation).

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The wheat samples were ground in a Udy mill through a 1-mm mesh screen to generate whole wheat flours used for analyses.

Instrumentation—Microspectrophotometry

The Zeiss UMSP8O microspectrophotometer installed in the Plant Research Centre of Agriculture Canada was employed for relative fluorescence intensity measurements and absorption and emission spectra (Fulcher et al, 1989). The microspectrophotometer is a modular system consisting of a Zeiss research microscope, a photometer head, monochromating devices including filter sets and grating monochromators, and a Hewlett-Packard minicomputer.

The microscope was equipped with a 100-W mercury burner (Osram HBO 100 W, Berlin) and a 10× photo eyepiece. Two software packages were employed: Lambda Scan and APAMOS. Two exciter-barrier filter combinations (FC) were used in this study consisting of FC 365 (exciter filter, G 365; beam splitter, FT 395; barrier filter LP 420) and FC 470 (exciter filter, BP 450-490; beam splitter, FT 510; barrier filter, LP 520). (Abbreviations: BP = band pass filter that transmits light limited by the range indicated; FC = filter combination; FT = chromatic splitter, which is a dichroic mirror acting as both exciter and barrier filters; LP = longwave pass filter which transmits light from a cutoff given by the wavelength with the transmittance 0.5. A more thorough explanation of filters used in fluorescence microscopy is given in Rost [1980].)

TABLE II
Data for Marketing Center Samples

Class and Location	Fluorescence Intensity[a]				Hardness	Protein (as-is)
	P_{470}	A_{470}	P_{365}	A_{365}		
Durum						
Grand Forks, ND	36.0	0.456	54.8	0.268	115.7	13.0
Moscow, ID	34.5	0.475	53.8	0.276	112.6	12.5
Grand Forks, ND	37.8	0.436	56.1	0.258	110.3	12.9
Minneapolis, MN	36.7	0.447	55.5	0.263	114.2	12.6
Moscow, ID	39.3	0.417	57.7	0.245	113.7	13.3
Hard red spring						
Grand Forks, ND	27.1	0.584	41.9	0.388	75.9	14.3
Portland, OR	30.0	0.545	47.5	0.331	83.0	15.0
Portland, OR	31.3	0.530	50.2	0.310	79.9	14.8
Portland, OR	29.8	0.545	48.1	0.327	77.8	14.8
Grand Forks, ND	32.5	0.508	43.7	0.368	86.1	16.4
Hard red winter						
Portland, OR	32.2	0.514	49.7	0.314	74.5	12.7
Wichita, KS	33.0	0.506	49.3	0.320	68.4	12.2
Omaha, NB	32.0	0.515	47.9	0.330	71.7	13.7
Portland, OR	31.9	0.518	45.6	0.351	74.4	13.6
Portland, OR	30.2	0.536	47.5	0.332	75.1	13.1
Wichita, KS	34.9	0.477	48.9	0.322	72.5	12.4
Soft red winter						
Indianapolis, IN	25.7	0.620	43.7	0.369	28.9	10.7
St. Louis, MO	24.2	0.642	43.5	0.375	24.6	10.1
Indianapolis, IN	25.2	0.626	43.0	0.377	32.1	10.7
Toledo, OH	25.3	0.626	45.1	0.359	29.0	10.1
St. Louis, MO	24.8	0.636	45.1	0.357	22.9	10.2
Soft white winter						
Saginaw, MI	34.1	0.487	54.2	0.277	23.3	9.7
Olympia, WA	30.0	0.543	49.1	0.319	35.8	11.8
Olympia, WA	29.7	0.546	45.2	0.311	37.8	11.9
Toledo, OH	35.8	0.469	57.1	0.254	24.1	9.9
Portland, OR	36.1	0.461	53.8	0.280	28.4	11.6
Saginaw, MI	36.0	0.473	55.6	0.266	22.9	9.9
Saginaw, MI	35.0	0.476	55.0	0.271	24.6	9.8
Toledo, OH	33.9	0.490	54.4	0.274	24.4	9.9
Portland, OR	37.8	0.443	55.1	0.269	28.9	11.4
Portland, OR	38.4	0.434	55.4	0.267	30.0	11.6
Club						
Portland, OR	44.4	0.376	59.7	0.232	29.2	9.6
Olympia, WA	45.4	0.357	54.3	0.274	30.3	12.3
Olympia, WA	45.9	0.359	56.2	0.260	31.7	12.4
Portland, OR	51.2	0.310	61.0	0.224	26.8	9.7
Portland, OR	40.7	0.413	54.9	0.272	28.0	11.5

[a] P_{470}, A_{470}; Relative fluorescence intensity (percent and absorbance) using filter cube 470 (450-490 nm excitation). P_{365}, A_{365}; Relative fluorescence intensity using filter cube 365 (365 nm, excitation).

Spectra

Two characteristic spectra, excitation and emission, may be generated for any fluorescent compound. An excitation spectrum can be defined as the relative efficiency of exciting radiation to induce fluorescence in a compound when the exciting radiation is varied in increments and emission is held constant. Similarly, an emission spectrum is the relative fluorescence intensity emitted by a source when the emission monochromator is varied in increments and the exciting radiation is held constant (Guilbault 1973).

Excitation and emission spectra were collected on each of three tissue components of wheat to determine relative intensity differences of the various components when the emission or excitation wavelength was optimized for one component. Data were collected on whole wheat flour from the cultivar Fredrick; recognizable tissue components (pericarp, endosperm, and aleurone) were selected and measured.

Whole wheat flour was placed on a slide, spread out, and compressed with another glass slide. Twenty-five intensity measurements for each tissue component were obtained using Lambda Scan software and averaged. Measuring parameters included a 10× Olympus objective, a 0.32-mm spot size, and a 0.63-mm luminous field diaphragm. The 0.63-mm luminous field diaphragm was roughly twice the size of the measuring spot, which is recommended for microspectrophotometry (Piller 1977). The photometer amplifier was adjusted (instrument standardization) on a very bright spot for a specific tissue optimization so that values for other tissues could be compared in terms of relative fluorescence intensity. Light was focused onto the sample by a mirrored apparatus. All tissues were measured both at their own optimum and at the optima for other tissues.

Excitation spectra were generated with the emission monochromator set at a specific wavelength (set value). Similarly, emission spectra were obtained with the excitation monochromator set at a specific wavelength (set value). Each monochromator bandwidth was always adjusted to 20 nm for spectral scans and set values. The set values were determined by trial and error for a specific tissue and were chosen to be optimal for the tissue of interest. Emission and excitation spectra were generated for endosperm and aleurone optimization (Figs. 1 and 2).

The emission spectrum of pericarp was optimum using FC 470, therefore FC 470 was used in place of the excitation monochromater. Only emission spectra were generated for pericarp optimization.

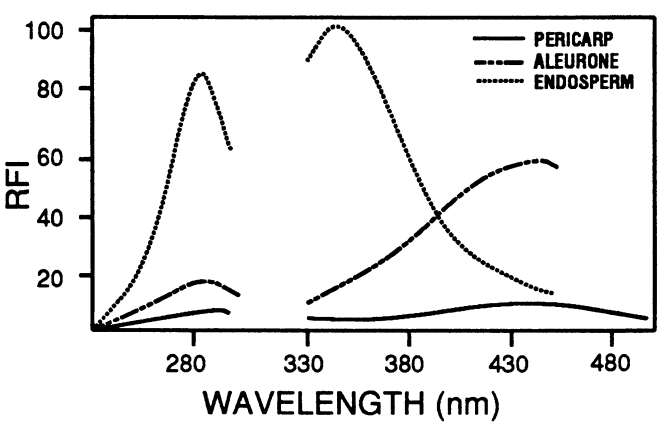

Fig. 1. Excitation (left) and emission (right) spectra at the optimum for endosperm tissue showing intensity differences between endosperm, aleurone, and pericarp tissues. The emission monochromator was set at 350 nm for absorption spectral scans and the excitation monochromator was set at 290 nm for emission spectral scans. **RFI** = relative fluorescence intensity.

Relative Fluorescence Intensity Scans

Intensity plot of a wheat kernel section. An unstained glycol methacrylate cross section (Irving et al 1988) of a wheat kernel was scanned using APAMOS software to generate an intensity profile. This procedure was done to visually illustrate instrument capability.

Prior to scanning the sample, the instrument had to be standardized to adjust the amplifier and gain by setting the intensity of the standard to 75%. Instrument standardization was accomplished on an aleurone cell wall spot that was the brightest in the field to be measured. The field to be scanned was selected by marking diagonal corners of the section, which allowed the software to generate a rectangle. The selected field included approximately six aleurone cells, endosperm, and pericarp. FC 365 (optimal for aleurone cell wall fluorescence) and a 10× objective were chosen for measurements. The selected field was then scanned and a relative fluorescence intensity profile was generated.

Mean intensity values of flours. The instrument was standardized using uranyl glass (UG 1 standard, Zeiss) attached to the objective at a specific distance. The use of a very stable standard such as uranyl glass allowed accurate, repeatable relative measurements of numerous samples over an extended time. As in the spectral plots and intensity profile, relative comparisons can be made when the samples are measured using identical parameters and standards.

Three grams of whole wheat flour was placed into a NIR spectrometer sample cup and compressed as for NIR. The cover was removed, and an area 5,000 × 5,000 μm was selected (by marking the center of the field) and scanned using APAMOS software. A specific number of points (2,352 pixels) within the scanned area was measured and each value stored. Two mean fluorescence intensity values were obtained: percent fluorescence (P) and absorption (A), which is a transformed value defined according to the equation:

$$A = \log[1/P(10^{-2})]$$

This transformation is similar to that of Beer's law, which defines the relationship between absorption and concentration, a nonlinear function (Freifelder 1982).

Emission intensities were obtained on whole wheat flours for five replicates using FC 470 and two replicates using FC 365. Mean intensity values for each replicate were averaged, and statistical analyses were executed on the resulting means.

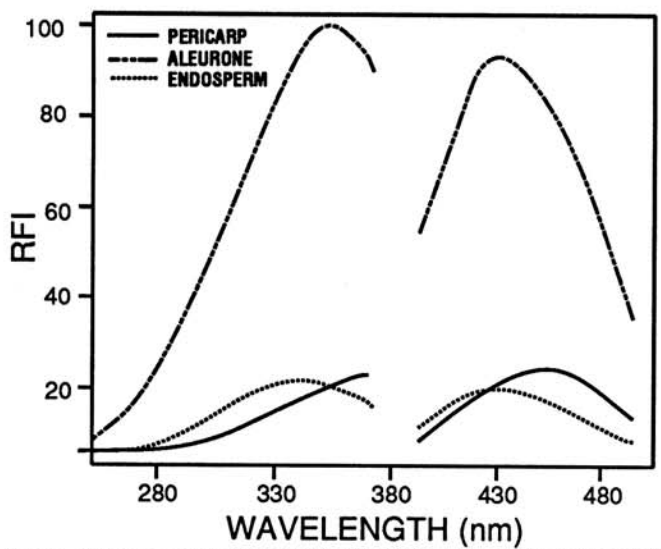

Fig. 2. Excitation (left) and emission (right) spectra at the optimum for aleurone tissue showing intensity differences between aleurone, endosperm, and pericarp tissues. The emission monochromator was set at 430 nm for excitation spectral scans and the excitation monochromator was set at 350 nm for emission spectral scans. **RFI** = relative fluorescence intensity.

Hardness

Hardness scores were obtained on whole wheat flours using a Technicon InfraAlyzer 500 and method 39-70 (AACC 1983).

Protein

Protein was measured by NIR using an equation generated from N × 5.7. Nitrogen data used for the calibration was obtained from N measured in a Carlo ERBA total combustion nitrogen analyzer.

Data Analysis

Data were analyzed using standard statistical procedures and Statistical Analysis System software (SAS Institute, Cary, NC, 1987) on an IBM XT personal computer.

Two sets of correlation analyses were computed on experimental data. One on all data combined and the other excluding the SWW and CLB. The purpose of this analysis was to determine the correlation between relative fluorescence intensity, hardness scores, and protein.

Experimental data were subjected to analysis of variance to determine if the wheat classes could be distinguished. Means different at the 5% level of probability were identified by calculating least significant difference values (Milliken and Johnson 1984).

Stepwise discriminant analysis was executed to determine the relative importance of each measurement in distinguishing between wheat classes. Once these analyses were obtained, discriminant analysis was done using equal or unequal variance to determine how well the wheat flours could be classified into the proper group based on a combination of measurements.

The two data sets (Tables I and II) were combined to provide sufficient numbers of each wheat class for analyses to improve the power of statistical tests comparing classes. Variability in number of samples within each class is accounted for in all analyses.

RESULTS

Excitation and Emission Spectra

Spectra were obtained using the cultivar Fredrick (a soft white wheat) to illustrate how each tissue component exhibits a spectrum at various wavelengths. An optimal wavelength may be selected for one tissue component; however, there is always an intensity contribution (interference) from other components making analyses somewhat difficult. All cultivars exhibited identical spectra, only intensity varied. Spectra were collected for each of three tissue components (endosperm, aleurone, and pericarp) at the optimum absorption and emission for each to show the contribution and/or interference attributable to each tissue when all were present in a sample such as whole wheat flour.

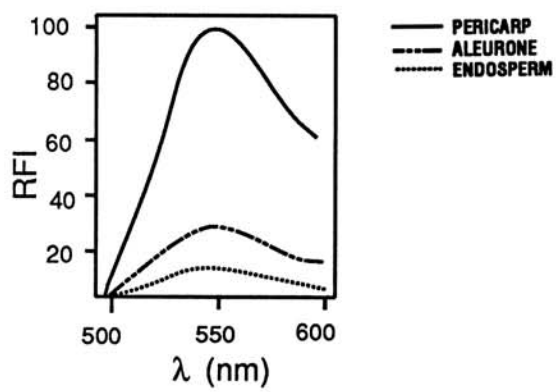

Fig. 3. Emission spectra of pericarp, aleurone, and endosperm tissues using filter combination 470 (450–490 nm excitation). **RFI** = relative fluorescence intensity.

Endosperm Optima

Endosperm tissue absorbed optimally at 290 nm with an emission peak at 350 nm (Fig. 1), therefore the excitation monochromator was set at 290 nm for generation of emission spectra. When aleurone cell walls, an indicator of aleurone tissue, were excited with 290-nm light, emission occurred at 350 nm at a reduced intensity level of about 20% of that for endosperm. The emission peak for aleurone tissue occurred at 430 nm. Pericarp emitted about 3% of the intensity of endosperm at 350 nm when excited with 290-nm light. A slight peak occurred at about 450 nm (Fig. 1) at the 290-nm excitation wavelength.

Excitation spectra for each tissue were generated with the emission monochromator set at 350 nm, optimal for endosperm. As expected, a peak occurred at 290 nm for endosperm tissue. Aleurone cell walls exhibited an absorption peak at 290 nm but had less than 20% of the intensity of that of the endosperm. Pericarp also exhibited approximately the same absorption peak but at about 8% of the intensity as that of the endosperm (Fig. 1).

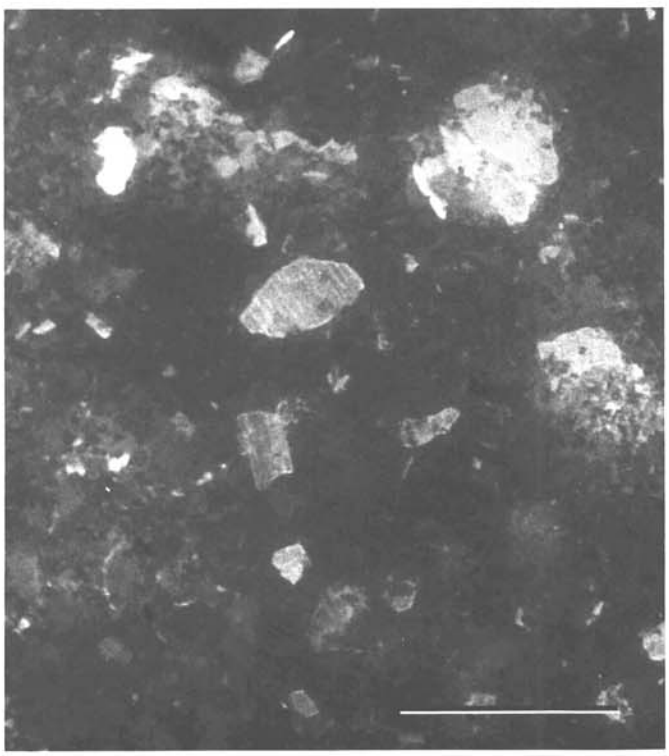

Fig 4. Micrograph of a whole wheat flour sample using filter combination 470 (450–490 nm excitation) to show relative fluorescence intensities of various tissue components in flour. Such a sample was scanned using APAMOS software to generate relative fluorescence intensity values. Magnification bar = 100 μm.

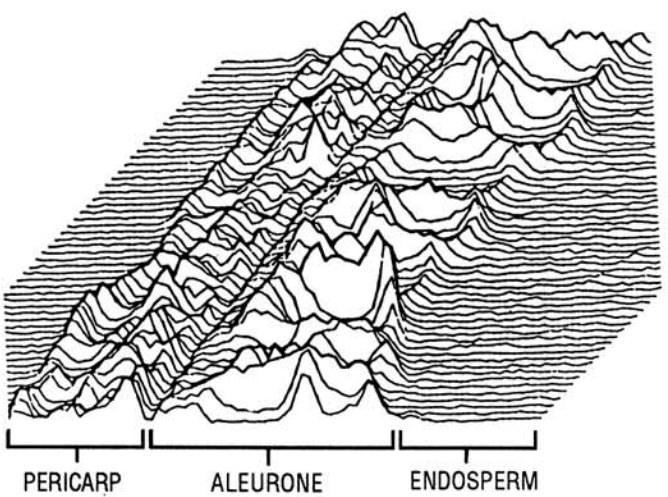

Fig. 5. An intensity plot of a section of a wheat kernel using filter combination 470 (450–490 nm excitation) and APAMOS software to illustrate fluorescence intensity differences. Note that the greatest intensity (highest peaks) occurs in the aleurone cell walls, and the least intensity occurs in the endosperm.

Aleurone Optima

Aleurone tissue absorbed optimally at 350 nm with an emission peak at 430 nm (Fig. 2). When the excitation monochromator was set at 350 nm, endosperm exhibited the same peak of emission as aleurone, 430 nm, with less than 20% of the intensity. Pericarp emission intensity was similarly less than 20% of that of the aleurone at 430 nm emission. An emission peak occurred at about 450 nm for pericarp when excited by light of 350 nm (Fig. 2).

An excitation spectrum of aleurone cell walls generated with the emission monochromator set at 430 nm yielded a peak at 350 nm as expected (Fig. 2). Endosperm tissue yielded the same absorption peak with an intensity of about 20% that of the aleurone. Similarly, pericarp absorbed light in this region but at reduced intensity and with the peak of absorbance shifted to a longer wavelength (Fig. 2).

Pericarp Optima

Separate excitation and emission monochromators were not used to generate spectra of pericarp tissue. Instead, FC 470, an exciter-barrier filter combination, was used to generate emission spectra. Because excitation in the range of 450–490 nm is built into the filter combination, excitation spectra are not meaningful, therefore only emission spectra are presented. The optimum emission maximum for pericarp occurred at about 550 nm. Aleurone and endosperm gave the same emission maximum but at reduced intensity; about 30 and 10% of that of pericarp for aleurone and endosperm, respectively (Fig. 3).

Relative Fluorescence Intensity Scans

Selected botanical components of the wheat caryopsis may be distinguished visually in whole wheat flour when viewed using fluorescence illumination (Fig. 4). Relative fluorescence intensities

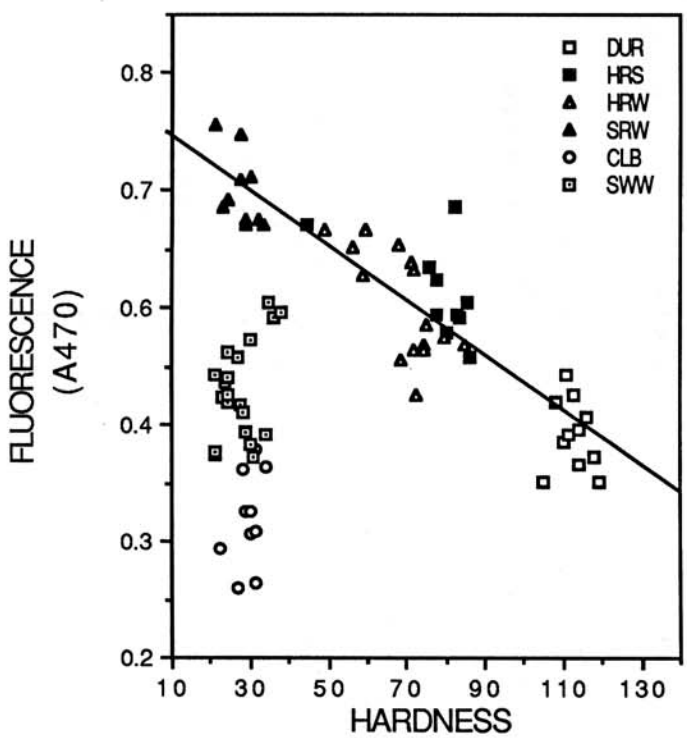

Fig. 6. Relative fluorescence intensity using filter combination 470 (450–490 nm excitation) and absorbance measurement (A_{470}) versus hardness of 77 wheat cultivars. The regression line was calculated for durum (DUR), hard red spring (HRS), hard red winter (HRW), and soft red winter (SRW); $r = -0.907$.

may also be visualized in an intensity profile generated by scanning a sample such as a section of a wheat kernel (Fig. 5) using APAMOS software. Regions of both high and low intensity, indicated by peaks and valleys in Figure 5, may be averaged numerically to give a single intensity value for the entire measured area. This value is termed relative fluorescence intensity (RFI). RFI values were also obtained for whole wheat flour samples.

RFI and NIR

RFI, hardness, and protein data are presented in Tables I and II.

A scatter plot of absorption (A) using FC 470 (A_{470}) versus NIR hardness indicates a linear relationship with a high negative correlation ($r = -0.907$, Fig. 6 and Table III) between RFI and hardness of the red wheat and durum flours. Inclusion of white wheats (CLB and SWW) in the analysis decreased the correlation coefficient ($r = -0.056$, Table IV). Correlation coefficients for most other variables show a similar trend (Tables III and IV).

Relative fluorescence intensity data using FC 470 show significant differences between the means of wheat types except HRS and HRW (Table V) as determined by analysis of variance. Hardness data show significant differences between the means of all wheats except the soft wheats (CLB, SRW, SWW), and the protein data show a considerable degree of overlap (Table V).

Stepwise discriminant analysis selects variables from the most important (the most discriminating variable) to the least important that classifies observations into groups. It excludes those that no longer add significant information for classification. Assuming equal variance, the sequence of variables from most to least significant was hardness, A_{470}, P_{470}, and A_{365}. Protein and P_{365} were excluded and, therefore, do not contribute more to the classification groupings when variances are assumed to be equal.

Significant variables, as determined assuming equal variance, were then subjected to discriminant analysis using both equal and unequal variance (Table VI). When unequal variance was assumed as part of the discriminant function, better classification resulted and 100% of the CLB, DUR, HRW, and SRW; 90% of HRS; and 85% of SWW were classified properly (Table VI). When protein and P_{365} were included, assuming unequal variance, only 6.3% of the observations were misclassified. This 6.3% represents one observation (Bennett), an HRW classified by discriminant analysis as HRS (Table VII). Therefore, combining all three variables and using a discriminant function for classification, in this instance, allows better classification than combining only two variables (fluorescence and hardness in Table VI, or protein and hardness in Table VIII) or considering variables alone (Table IX).

Since the cultivar Oslo has been considered an anomaly and has been variously classified by other centers, it is somewhat surprising that Bennett was the anomaly in this study. Analyses were run without Oslo, and the results remained the same when all variables (fluorescence, protein, and hardness, Table VII) were considered. Excluding Oslo when only protein and hardness were considered resulted in slightly better classification, with 100% of HRS and 87.5% of HRW being properly classified as opposed to 90 and 75%, respectively (Table VIII) when all data were included. All other classifications are identical to those in Table VIII.

TABLE III
Correlations Between Variables Within Four Wheat Classes[a]

	Variable					
Variable	A_{470}	P_{470}	Hardness	A_{365}	P_{365}	Protein
A_{470}	1.00[b] 0.0[c]					
P_{470}	−0.997 0.0001	1.00 0.0				
Hardness	−0.907 0.0001	0.887 0.0001	1.00 0.0			
A_{365}	0.859 0.0001	−0.881 0.0001	−0.734 0.0001	1.00 0.0		
P_{365}	−0.481 0.0006	0.494 0.0004	0.409 0.0043	−0.659 0.0001	1.00 0.0	
Protein	−0.324 0.0262	0.301 0.0397	0.456 0.0013	−0.155 0.2982	−0.170 0.2540	1.00 0.0

[a] Durum, hard red spring, hard red winter, and soft red winter.
[b] Pearson coefficient.
[c] Prob > |R|, the significance probability of the correlation under the null hypothesis that the correlation is zero.

TABLE IV
Correlations Between Variables Within Six Wheat Classes[a]

	Variable					
Variable	A_{470}	P_{470}	Hardness	A_{365}	P_{365}	Protein
A_{470}	1.00[b] 0.0[c]					
P_{470}	−0.990 0.0001	1.00 0.0				
Hardness	−0.056 0.6285	−0.020 0.8660	1.00 0.0			
A_{365}	−0.841 0.0001	−0.815 0.0001	−0.008 0.9473	1.00 0.0		
P_{365}	−0.444 0.0001	0.426 0.0001	0.025 0.8323	−0.610 0.0001	1.00 0.0	
Protein	−0.023 0.8417	−0.010 0.9324	0.486 0.0001	0.083 0.4712	−0.312 0.0058	1.00 0.0

[a] Durum, hard red spring, hard red winter, soft red winter, soft white winter, club.
[b] Pearson coefficient.
[c] Prob > |R|, the significance probability of the correlation under the null hypothesis that the correlation is zero.

TABLE V
Means and Standard Deviations (SD) of Wheat Classes

		A_{470}[a]		P_{470}[b]		A_{365}[c]		P_{365}[d]		Protein[e]		Hardness[f]	
Class[g]	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DUR	11	0.442 d[h]	0.030	37.17 b	2.48	0.258 d	0.026	55.08 a	5.04	13.08 ab	1.83	112.69 a	4.24
HRS	10	0.564 b	0.040	28.72 d	2.64	0.364 a	0.044	46.05 c	6.58	13.86 a	1.61	77.51 b	12.11
HRW	16	0.554 b	0.045	29.53 d	2.95	0.357 a	0.026	45.91 c	4.27	12.03 bc	1.25	68.67 c	8.99
SRW	10	0.650 a	0.031	23.82 e	1.84	0.369 a	0.012	48.21 bc	5.62	10.93 c	0.92	27.68 d	3.94
SWW	20	0.480 c	0.041	34.84 c	3.25	0.279 bc	0.024	52.38 a	4.84	11.07 c	1.57	27.54 d	5.12
CLB	10	0.369 e	0.041	44.92 a	4.18	0.266 cd	0.021	52.63 ab	4.41	11.51 c	1.55	29.50 d	3.30

[a] A_{470}: Relative fluorescence intensity (absorbance) using filter cube 470.
[b] P_{470}: Relative fluorescence intensity (percent) using filter cube 470.
[c] A_{365}: Relative fluorescence intensity (absorbance) using filter cube 365.
[d] P_{365}: Relative fluorescence intensity (percent) using filter cube 365.
[e] Protein: Near-infrared protein calibrated by ERBA × 5.7.
[f] Hardness: Near-infrared reflectance spectroscopy data.
[g] DUR = Durum, HRS = hard red spring, HRW = hard red winter, SRW = soft red winter, SWW = soft white winter, CLB = club.
[h] Values followed by different letters are significantly different at the 5% level.

DISCUSSION

Excitation and emission spectra of selected botanical components of the wheat caryopsis reveal striking differences enabling differentiation and quantification (Jensen et al 1982). Proteins exhibit intrinsic fluorescence in the ultraviolet (UV) region of the spectrum, which has been shown to be primarily due to the presence of tryptophan; however, tyrosine and phenylalanine also fluoresce in the UV. Excitation and emission maxima of 290 and 350 nm, respectively is characteristic of tryptophan (Chen 1973). Thus, the intrinsic fluorescence of wheat endosperm (Fig. 1), is probably due to tryptophan, although there could be a contribution from tyrosine as well.

Aleurone cell walls, which contain high concentrations of ferulic (4-hydroxy-3-cinnamic) acid, fluoresce in the blue region of the spectrum (Fulcher et al 1972). Optimal excitation for aleurone cell walls occurred at 350 nm and emission occurred at 430 nm. Emission spectra of individual tissues using FC 365 is similar to those shown in Figure 2. This is explained by the overlap that occurs between the excitation monochromator settings (at 350 nm) and FC 365, because the bandwidths in either case are approximately 20 nm.

Exciter-barrier filter combinations were more practical than using an excitation monochromator for repeated measurements. The emission monochromator functioned as a barrier filter and the excitation monochromator as an exciter filter in the generation of Figures 1–3. No barrier filter occurred between excitation monochromator and the observer. Therefore, when a sample was illuminated with light at 400 nm or longer (i.e., visible light), the excitation and emission light interfered and the observer saw nothing. Since focusing and positioning the sample are important, several adjustments are necessary before measurements can proceed. Use of a filter combination also eliminates the necessity for a complicated microspectrophotometer with two monochromators. Thus, considerable simplification is realized by using filter combinations in the measurement of samples to generate intensity plots or RFI values.

An intensity plot of a wheat section (Fig. 5) using FC 365 illustrates that all tissues fluoresce to some degree at this excitation wavelength as well as at other excitation wavelengths (Figs. 1–3). Sufficient differences in intensity occur, however, making it possible to differentiate between tissues. The intensity plot (Fig. 5) can also be used as a control measure for instrument calibration and noise in the system. Intensity plots were generated on whole wheat flours to set measurement parameters for RFI values.

TABLE VI
Discriminant Analysis: Fluorescence and Hardness[a]

Actual Classification	Classification[b] from Analysis					
	DUR	HRW	HRS	SRW	SWW	CLB
Equal variance						
DUR	100.0[c]					
HRW		75.0	18.7	6.3		
HRS		10.0[d]	90.0			
SRW				100.0		
SWW					90.0	10.0
CLB					10.0	90.0
Unequal variance						
DUR	100.0					
HRW		100.0				
HRS		10.0	90.0			
SRW				100.0		
SWW					85.0	15.0
CLB						100.0

[a]Fluorescence intensity at 365 nm (variable A_{365}) and 450–490 nm (variable A_{470}) excitation wavelengths; Hardness as determined by near-infrared reflectance spectroscopy.
[b]DUR = Durum, HRW = hard red winter, HRS = hard red spring, SRW = soft red winter, SWW = soft white winter, CLB = club.
[c]Percent correctly classified.
[d]Percent misclassified.

TABLE VII
Discriminant Analysis (unequal variance): Fluorescence, Hardness, and Protein[a]

Actual Classification	Classification[b] from Analysis					
	DUR	HRW	HRS	SRW	SWW	CLB
DUR	100.0[c]					
HRW		93.7	6.3[d]			
HRS			100.0			
SRW				100.0		
SWW					100.0	
CLB						100.0

[a]Fluorescence intensity at 365 nm (variables A_{365}, P_{365}) and 450–490 nm (variables A_{470}, P_{470}) excitation wavelengths; hardness and protein as determined by near-infrared reflectance spectroscopy.
[b]DUR = Durum, HRW = hard red winter, HRS= hard red spring, SRW = soft red winter, SWW = soft white winter, CLB = club.
[c]Percent correctly classified.
[d]Percent misclassified.

TABLE VIII
Discriminant Analyses: Protein and Hardness[a]

Actual Classification	Classification[b] from Analysis					
	DUR	HRW	HRS	SRW	SWW	CLB
Equal variance						
DUR	100.0[c]					
HRW		75.0	18.7[d]			6.3
HRS		10.0	80.0			10.0
SRW				70.0	20.0	10.0
SWW				45.0	10.0	45.0
CLB				40.0	20.0	40.0
Unequal variance						
DUR	100.0					
HRW		75.0	25.0			
HRW		10.0	90.0			
SRW				90.0		10.0
SWW				40.0	40.0	20.0
CLB				60.0		40.0

[a]Protein and hardness as determined by near-infrared reflectance spectroscopy.
[b]DUR = Durum, HRW = hard red winter, HRS = hard red spring, SRW = soft red winter, SWW = soft white winter, CLB = club.
[c]Percent correctly classified.
[d]Percent misclassified.

TABLE IX
Discriminant Analysis: Unequal Variance

Actual Classification[a]	Classification[a] from Analysis					
	DUR	HRW	HRS	SRW	SWW	CLB
Hardness[b]						
DUR	100.0[c]					
HRW		87.5	12.5			
HRS		10.0[d]	90.0			
SRW				20.0	20.0	60.0
SWW				30.0	35.0	35.0
CLB				10.0	10.0	80.0
Fluorescence[e]						
DUR	100.0[c]					
HRW		62.5	25.0	6.3	6.3	
HRS		30.0[d]	50.0		20.0	
SRW				100.0		
SWW	5.0	5.0	10.0		75.0	5.0
CLB					10.0	90.0

[a]DUR = Durum, HRW = hard red winter, HRS = hard red spring, SRW = soft red winter, SWW = soft white winter, CLB = club.
[b]Hardness as determined by near-infrared reflectance spectroscopy.
[c]Percent correctly classified.
[d]Percent misclassified.
[e]Fluorescence intensity at 365 nm (variable A_{365}) and 450–490 nm (variables A_{470} and P_{470}) excitation wavelengths.

RFI values generated using FC 365 are relative measures of aleurone cell wall material as is FC 470 a measure of pericarp. Other tissue components contribute to this measurement to a lesser degree (Figs. 1–3) resulting in interference of fluorescence signals. This interference phenomenon was also observed by Jensen et al (1982). Fluorescence measurements are most useful when the particular autofluorescent component of interest is in a relatively pure medium such that interference is minimal. Such a system could be used readily in monitoring wheat mill streams as was done by Pussayanawin et al (1988), where ferulic acid content was monitored chemically and using autofluorescence to measure aleurone (bran) content of flour.

Most measurements of hardness of wheat including NIR seem to be related to particle size (Pomeranz et al 1985). Because of the spot measuring characteristics of the microspectrophotometer using APAMOS software, perhaps particle size has less effect on the average intensity value obtained.

The high correlation between hardness and A_{470} on durum and red wheats ($r = -0.907$, Fig. 5) suggests a relationship between pericarp and hardness; however, when white wheats were included in the analysis, the correlation was reduced ($r = -0.05$). The discrepancy between reds and whites may be due to differences in pericarp pigmentation and other factors such as refractive or scattering properties. Hard white wheats were not included in the analyses (because they are not typical of U.S. commercial wheats) and could have provided key information on the influence of pericarp pigmentation on fluorescence. The important point to remember is that several factors contribute to a particular attribute, and we are measuring complex genetic differences that we don't understand. No single measurement is likely to give an answer to the classification problem; however, a combination of measurements taken together and analyzed by a discriminant function may allow a better classification scheme. This scheme is not immediately adaptable for use at inspection sites due to the complexity of the instrumentation and analyses, but it may be useful as a reference method.

Future studies will include in-depth analysis of spectral characteristics of various tissue components and how these differences may contribute to wheat classification.

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