NOTE

Measurement of Durum Pigment with a
Near-Infrared Instrument Operating in the Visible Range

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Semolina color is an important constituent of pasta quality. The yellow color is due to the presence of xanthophyll and other related compounds throughout the endosperm (Lepage and Sims 1968). Because of the important relationship of color with end-use quality, considerable effort has been expended in breeding programs for durum wheat (*Triticum turgidum* L. var *durum*) to measure pigment characteristics. Three methods of measurement have commonly been employed: 1) visual comparisons with standard samples, 2) colorimetry following solvent extraction, and 3) light reflectance measurements. These methods were reviewed by Johnston et al (1980).

Near-infrared reflectance (NIR) spectrophotometers are rapidly becoming common in cereal quality laboratories. Most chemical constituents of cereals, like all agricultural products, absorb radiation in the near-infrared range (780–2,600 nm), and NIR instruments have been developed and successfully used to measure cereal constituents including protein, kernel hardness, and moisture (Williams and Thompson 1978, Williams 1979). The latest generation of NIR instruments has extended the spectral range to include the visible region (380–780 nm). Reflectance spectrophotometers that span the visible range have long been available and have been used to measure pigment in durum wheat (Johnston et al 1980). However, these instruments lack the computerization and convenience of the new scanning NIR instruments. Furthermore, combining both the visible and near-infrared regions in one instrument makes a vast improvement in efficiency related to instrumental, sampling, and analytical costs. Because many quality characteristics are associated with visible characteristics such as color or brightness, the visible region may soon prove to be as useful as the near-infrared region.

In our durum breeding program we are currently measuring protein content by NIR. Our objective was to determine whether a scanning NIR instrument, operating in the visible range, could accurately measure pigment content.

MATERIALS AND METHODS

Based on an initial screening for pigment, a calibration-validation set of 53 wheat samples (200 g each) was selected from the collection of durum wheat germ plasm at the Agriculture Canada Research Station at Swift Current, Saskatchewan. The sample set was selected to cover a broad range of genetic diversity. It consisted of 51 genotypes including: seven registered Canadian cultivars or germ plasms (Kyle, Medora, Wakooma, Arcola, Plenty, DT637, and DT369); lines from the collections of the International Center for Agricultural Research in Dry Areas (ICARDA) and the International Maize and Wheat Improvement Center (CIMMYT); and crosses between Canadian cultivars and ICARDA and CIMMYT lines. The seed was harvested from plots grown at Swift Current, Saskatchewan, in 1990. Air cleaning was used to remove chaff. The wheat samples ranged from 4 to 11 ppm pigment and provided a relatively flat distribution over this range (although one sample of >12 ppm was also included). A second set of 28 samples, derived from crosses between Kyle and ICARDA lines, was collected from plots grown in Swift Current, Saskatchewan, in 1991. This set was used to validate equations and ranged in pigment content from 4 to 10 ppm.

Because we are currently using NIR in our breeding program to measure several quality-related characteristics on each sample, we have chosen to standardize sample moisture contents before grinding and before spectra collection. This procedure has been adopted by other breeding programs (Allen et al 1986). It minimizes problems associated with calibrating each method for potential direct effects of moisture content on the spectra and minimizes potential particle-size errors that may result from grinding at various moisture contents. The lengthy equilibration procedure could probably be eliminated if a calibration sample set were selected to correct for moisture differences.

Whole-grain samples were equilibrated to approximately 9% moisture by placing them in a controlled environment room (22° C and 40% rh) for one week. Samples were ground using a cyclone mill (Udy Corp., Fort Collins, CO) fitted with a regulated feed mechanism and a 1-mm screen. Ground samples were then returned to the controlled-environment room for one week to re-equilibrate samples to 9% moisture.

The pigment analysis was carried out according to the procedure currently being used by the Wheat Quality Laboratory (Winnipeg, MB) to evaluate early-generation lines within the Canadian durum-breeding programs. This procedure is identical to AACC approved method 14-50 (AACC 1983), with a 16-hr extraction, except that 85% ethanol replaced water-saturated *n*-butanol. To ensure that results were comparable with the two solvents, 25 wheat samples were extracted using both methods. The laboratory analyses with ethanol were repeated five times on separate days. Each day, a single subsample of each of the 53 samples was extracted. The absorbance (435 nm) was then determined on duplicates from the single extract of each subsample. Absorbances from the duplicates were averaged to provide a single pigment value (dry weight basis) for each sample for each of the five days; these values were used to calculate the standard error of the laboratory (SEL) (Mark and Workman 1991). Values for the five days were further averaged to provide a single value for each sample that was used in the regression procedures.

Spectra consisted of log $1/R$ values (where R stands for reflectance) at 2-nm increments over the 400- to 2,500-nm range from a model 6500 scanning spectrophotometer (NIRSystems, Silver Spring, MD) using the rotating sample cups (36 mm window diameter). Spectra collection, manipulation, and wavelength selection were carried out using the ISI NIRS2 software (NIRSystems). Because the raw spectra are very susceptible to particle size interferences (Hruschka 1987), a first-derivative transformation was applied before wavelength selection. The 1-4-4 math treatment, as recommended in the ISI NIRS2 software, was used to transform spectra to the first derivative. (This smooths the spectra by replacing each data point with a moving mean of four data points and then calculates the first derivative as a moving difference of four data points.) Stepwise regression (Kleinbaum et al 1988) was used to select appropriate wavelengths.

RESULTS AND DISCUSSION

Chemical analyses based upon organic solvent extraction presents a health and fire risk. The pigment extraction process of the Wheat Quality Laboratory, Winnipeg, uses 85% ethanol, instead of water-saturated *n*-butanol (AACC 1983), because of the lower health hazards associated with long-term exposure to ethanol rather than butanol (Occupational Safety and Health Data Sheets, Canada Safety Council, Ottawa, ON). We compared both
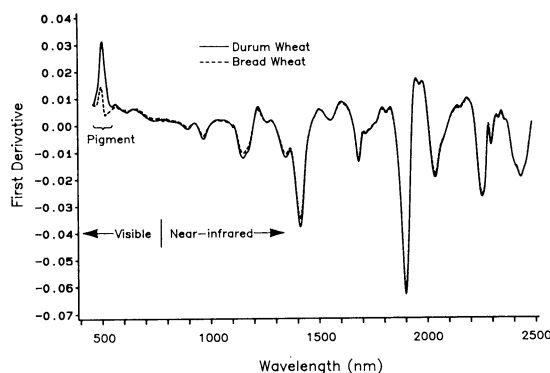
Fig. 1. First derivative of the visible/near-infrared reflectance spectra of a durum and a bread wheat.

Fig. 2. Near-infrared (NIR) versus laboratory pigment values for the calibration sample set. (The equation was based on the first derivative at 498 nm using all 53 samples.)

solvent systems and found an R^2 of 0.96 ($n = 25$). A t test also indicated that the absorbance values were not significantly different between the two solvents ($P = 0.27$); so the same conversion factor (AACC 1983) is valid for both solvents. Lier and Lacroix (1974) and Lepage and Sims (1968) have also demonstrated that numerous alcohol-based solvent systems can be used for the extraction of durum pigments. Since the yellow color is due to a mixture of compounds, and it is not known if the mixture is constant for all durum genotypes, these methods provide only a relative measure of pigment content (AACC 1983).

NIR procedures are usually calibrated to a laboratory (reference) method that they are intended to replace. It is important, therefore, to obtain accurate reference method data as well as an indication of the day-to-day variation typical of the reference method. Repetition of the laboratory pigment analyses five times on separate days improved mean values for each sample, and the SEL provided a better indicator of the reproducibility of the laboratory method over time. The resultant SEL was 0.56 ppm pigment.

The spectrum (first derivative) of a durum wheat sample is shown in Figure 1. The large absorption near 500 nm is due to the presence of the pigments. The absorption values reported here are shifted slightly toward longer wavelengths, compared to those observed by Sims and Lepage (1968), because of data smoothing and the algorithm used to calculate the first derivative. Bread (hexaploid) wheat (*Triticum aestivum* L.) is low in these pigments; a spectrum is included in Figure 1 for comparison with the durum (tetraploid) wheat. The major spectral difference between the two species is in the region of pigment absorption.

A prediction equation was first developed using least-squares regression analysis based on all 53 samples grown in 1990. When limited to one wavelength and first derivative data treatment, the selected wavelength was 498 nm ($R^2 = 0.94$, standard error of calibration [SEC] = 0.53 ppm). The plot of the predicted values based on this single wavelength is shown in Figure 2. When allowed to select two wavelengths, the program included 2,248 nm, which increased the R^2 to 0.97 and reduced the SEC to 0.32.

The 53 samples were randomly divided into two sets of 27 and 26 samples to cross-check validation of equations. When limited to a single regression term, 498 nm was selected for both sets and the R^2 values were 0.95 and 0.94. When the equations developed from one set were used to predict values for the other set, and vice versa, the R^2 values were both 0.94. However, if two terms were selected, each set selected a different second wavelength in the near-infrared region, which may or may not be related to pigment. Therefore, we have limited our equation, at present, to a single term at 498 nm. The broad diversity of genotypes within the 53-sample set means that the equation should be relatively robust with respect to genotypes. However, to determine the influence of environment, the single-term equation was further tested using a validation set of 28 samples that were grown in 1991. For these samples the R^2 between the NIR and laboratory method was 0.87 and the SEC was 0.77. These results suggest that the environmental effects on pigment content may not be
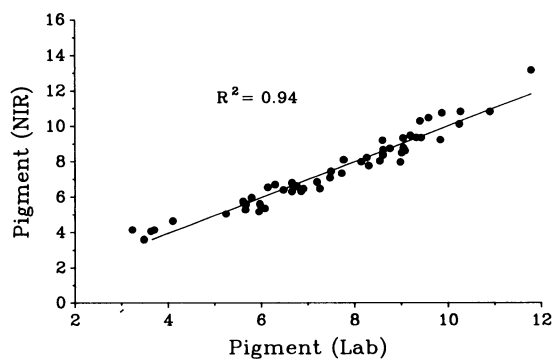
large, although more robust equations would probably result from including samples from several environments.

An indication of the variability of the NIR method, independent of sampling variability, was obtained by collecting spectra from 25 samples on two consecutive days without unloading the cells. The standard error of the difference was 0.07 ppm pigment. When the 25 samples were repacked with fresh sample before spectra collection on two consecutive days, the standard error of difference increased to 0.45 ppm.

These results indicate that pigments can be measured very easily and accurately in a diverse group of durum genotypes using the visible range of the new generation of NIR instruments. As is true with most NIR methods, researchers planning to measure durum pigment by NIR should develop a set of standard samples that covers their specific genetic diversity and growing conditions. The major advantages of the method are: 1) minimal additional sampling and processing time and cost in breeding programs currently using NIR instruments covering the visible range, and 2) elimination of the health and environmental hazards associated with organic solvent extractions.

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