Determination of Chlorophyll in Ground Rapeseed Using a Modified Near Infrared Reflectance Spectrophotometer

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A rapid and accurate method has been developed for the analysis of chlorophyll in ground rapeseed using a modified near infrared filter instrument. Ground rapeseed was scanned with a Cary 17 spectrophotometer, and optimal wavelengths for chlorophyll prediction (674, 696 nm) were selected from the visible wavelength region by multiple linear regression analysis. Six Dickey-john Instalab 600's were modified to analyze chlorophyll in ground rapeseed by replacing two standard near infrared filters with two filters having central wavelengths of 674 and 696 nm. Calibration equations incorporating data from three wavelengths (674, 696 and 2100 nm) had an average multiple correlation coefficient (R) of 0.980 and standard error of estimate (SEE) of 3.1 ppm. Instrument prediction of chlorophyll agreed well with reference solvent extraction analyses (standard error of prediction, SEP = 3.0 ppm). A universal calibration equation developed for the determination of chlorophyll in ground rapeseed was tested with several modified instruments and performed well.

Crude oils extracted from immature rapeseed are often dark in color due to the presence of the chlorophyll-related photosynthetic pigments. In addition to imparting an undesirable color to rapeseed oil, chlorophyll pigments may also affect the rate of hydrogenation (1) and accelerate oxidation (2,3), resulting in a prematurely rancid product. Chlorophyll pigments are difficult to remove by conventional alkali treatment and bleaching during processing (4), and their removal increases the cost of refining. It is therefore important that a practical and reliable method for determining chlorophyll in rapeseed be established.

The Canadian oilseed industry presently uses the percentage of distinctly green seeds and overall sample color as a guide to chlorophyll content. However, "percentage green seed" correlates poorly with the level of chlorophyll in the seed or extracted oil (5), and there is growing criticism within the industry that the current visual grading system is too subjective. The Swedish Oilseed Association has introduced a grading procedure for rapeseed which requires extraction of oil from the seed (6), followed by determination of chlorophyll content of the oil by a modification of the official method of the Americal Oil Chemists' Society (AOCS). The AOCS method is based on the absorbance of chlorophyll near 670 nm, corrected for background on each side of the peak. While this method has been used routinely in many extraction and refining plants, there is a growing interest within the industry in the development of a simpler and less time-consuming method.

Although near infrared (NIR) reflectance techniques are less than satisfactory for prediction of chlorophyll in whole rapeseed (7), studies have shown that direct reflectance measurements of ground rapeseed at the visible wavelength of 670 nm (i.e., without oil extraction of the seed) can be used to measure chlorophyll with reasonable accuracy (8). This paper describes the development of a reflectance technique for analyzing chlorophyll in ground rapeseed using a modified NIR filter instrument.

MATERIALS AND METHODS

Modified solvent extraction method for chlorophyll analysis. Chlorophyll content was determined in quadruplicate by the solvent extraction method of the Swedish Oilseed Association (6) as modified by Daun (8), with the following changes. The calibration curve was prepared with a primary standard of crystalline chlorophyll a (*Anacystis nidulans*, Fluka Chemical Corp.). Chlorophyll (155 μg) was dissolved in 10 ml acetone, and purity was determined by comparing ratios of the "red" and "blue" absorption maxima as described by Strain et al. (9); the concentration of chlorophyll in solution was calculated using reported extinction coefficients. Appropriate volumes of this stock solution were pipetted into tubes, dried under a stream of nitrogen, and made up to 5.0 ml with 3:1 heptane/ethanol (v:v) containing 2.5% colorless refined rapeseed oil to give standards ranging from 0.3 to 2.5 ppm chlorophyll. Absorbance was read on a Cary 17 spectrophotometer and corrected for optical density of the solvent according to the formula $A_{corr} = A_{665} - (A_{710} + A_{630})/2$ (note: chlorophyll in heptane/ethanol had a maximum absorbance at 665 nm on the Cary 17). To measure chlorophyll content of the rapeseed samples, freshly ground seed (2 g) was weighed into stainless steel centrifuge tubes, and ball bearings and 30 ml of heptane/ethanol extract solvent were added. Tubes were shaken for one hr, and the absorption of the filtrates was measured at 710, 665 and 630 nm. Equivalent extractions could be obtained by homogenizing two g seed and 30 ml heptane/ethanol in a 50-ml Erlenmeyer flask for 60 sec with a Polytron homogenizer (Brinkman Instruments) fitted with a PT 10 ST generator. The chlorophyll content of the seed was calculated as follows:

$$\text{chl in seed (ppm, as is moisture)} = 11.408 \times A_{corr} + 0.008 \times \frac{30 \text{ ml}}{\text{wt seed in g}}$$

Optimal wavelength selection for chlorophyll in ground rapeseed. A total of 94 samples were selected from the Grain Research Laboratory's 1982 new crop survey of Western Canada. Selections included 26 samples of No. 1 Canada Rapeseed (1CR), 67 samples of 2CR and 1 sample of 3CR. Moisture content of the sample ranged from 4.5 to 5.0%, while chlorophyll con-

tent ranged from 1.7 to 48.3 ppm (seed basis, as is moisture). It should be noted that in 1985, the Canadian General Standards Board set 30 ppm as the maximum chlorophyll content for top grade crude rapeseed oil, which corresponds roughly to 25 ppm chlorophyll in the seed. Each sample (8 g) was ground in a GRL rapeseed grinder and loaded in a cylindrical sample holder (1 cm deep, 6.5 cm diameter) faced with an Infrasil cover. Reflectance data was collected at 900 wavelengths (600 to 2400 nm region at 2 nm intervals) with a Cary 17 spectrophotometer controlled by a PDP 11/34 minicomputer (10). Reflectance readings were recorded as apparent absorbance (A'), where A' = log (1/reflectance), and data points were smoothed with a 9-point quartic convoluting function (11). Wavelengths that best predicted chlorophyll in ground rapeseed (i.e., that gave regression equations having a high multiple correlation coefficient R and low SEE) were selected by stepwise multiple linear regression analysis using a PDP-11 version of the BMPD-77 Statistical Software Package (Software Development Inc., Middlebury, Vermont).

Modification and calibration of the Dickey-john GAC III's. Six Dickey-john Instalab 600 NIR Product Analyzers were modified to measure chlorophyll in ground rapeseed by replacing NIR filters in positions F0 and F1 (2310, 2230 nm) with two narrow bandpass interference filters having central wavelengths of 674 and 696 nm and half peak bandwidths of 11±2 nm (Infrared Industries Inc., Orlando, Florida). Instruments were calibrated with 66 rapeseed samples selected from various sources, including 1983 carlot and cargo unloads at Vancouver and Thunder Bay terminal elevators. Moisture content of the samples ranged from 6.0 to 7.0%, while chlorophyll content ranged from 1.9 to 59.2 ppm (seed basis, as is moisture). Samples (15 g) were ground in a Braun model KSM2 coffee grinder for two 15-sec bursts (30 sec grinding in total), with stirring of the sample inside the grinder between bursts. Two open sample cups were loaded to overflowing, patted gently and levelled with a spatula. Log readings were collected for each sample cup and averaged. One-half of the samples (odds, N = 33) were used to develop a calibration equation, while the remaining samples (evens, N = 33) were used as unknowns to test the accuracy of chlorophyll prediction.

RESULTS AND DISCUSSION

Calibration of the reference solvent extraction method. Both chlorophyll a and b are found in plants. In rapeseed, the major portion of the pigments are of the a-type. During the decomposition of chlorophyll, the porphyrin ring structure will release bound Mg, leaving behind pheophytin (12). While industrially processed rapeseed oils often contain high levels of pheophytins (3,13), it has been shown that heptane/ethanol extracts of rapeseed contain mainly chlorophyll rather than pheophytin (14). Recent HPLC studies of rapeseed pigments in our own laboratory support the finding that only traces of pheophytin are present in laboratory extracts (unpublished data).

Prior to 1983, the standard chlorophyll curve for the solvent extraction method at the GRL was prepared by extracting oil from a rapeseed sample with heptane/ethanol solvent, desolventizing and drying, and then testing this lab-extracted oil for chlorophyll by AOCS official method Cc 13d-55 (15). Dilutions of this oil were then made in heptane/ethanol. Preparing a standard curve in this way, however, does not consider the possibility that chlorophyll could be converted to pheophytin as a result of this relatively harsh treatment. Although chlorophyll a and pheophytin a have absorption maxima at similar wavelengths, the molar extinction coefficient (i.e., absorptivity) of pheophytin a is approximately 60% that of chlorophyll a (16). This would mean that if any chlorophyll was broken down to pheophytin in a laboratory-extracted oil during desolventizing and/or drying, then a standard curve prepared from this oil would underestimate the amount of chlorophyll in a sample extract. For this reason, the GRL has now adopted the procedure of calibrating the solvent extraction method with a primary standard of crystalline chlorophyll a.

Optimal wavelength selection for chlorophyll in ground rapeseed. Reflectance spectra of typical ground rapeseed samples are shown in Figure 1. Two wavelengths from the visible region of the spectrum, 674 and 696 nm, were selected by stepwise regression analysis as the best predictors of chlorophyll in ground rapeseed (Table 1). Entry of additional wavelengths from the NIR region of the spectrum (1712, 2266 nm) improved the correlation and standard error of regression line only slightly.

Chlorophyll analysis using the modified Dickey-john GAC III. Near infrared reflectance instruments

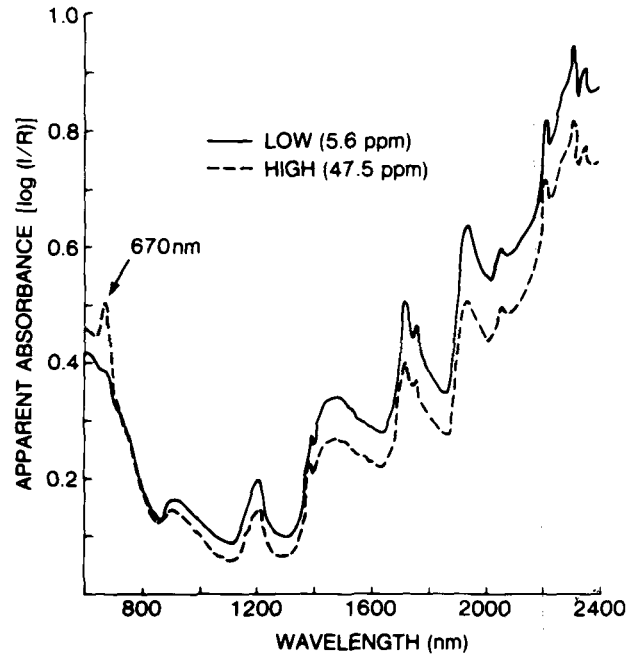

FIG. 1. Reflectance spectra of ground rapeseed samples containing high (47.5 ppm) and low (5.6 ppm) levels of chlorophyll (seed basis, as is moisture).

TABLE 1

Wavelength Selection for Chlorophyll in Ground Rapeseed Using the Cary 17

Step	Wavelengths selected (nm)	Calibration (N=94) R	SEE
1	674	0.764	7.7
2	696	0.951	3.7
3	1712	0.961	3.3
4	2266	0.962	3.3

have been used for many years to determine the concentration of components such as protein, oil and moisture in grains and oilseeds. The Dickey-john Instalab 600 is equipped with six narrow bandpass filters which provide six simultaneous reflectance readings for each sample. Six such Dickey-john instruments were modified to analyze chlorophyll in ground rapeseed by replacing NIR filters provided for oil analysis (2310, 2230 nm) with two visible filters having central wavelengths of 674 and 696 nm. Stepwise multiple linear regression analysis of reflectance readings for the odd-numbered samples (N = 33) showed that a combination of the 674, 696 and 2100 nm wavelengths gave the best estimation of chlorophyll. Calibration equations incorporating reflectance data from these three wavelengths had an average R of 0.980 and SEE of 3.1 ppm (Table 2). The linear regression plot for calibration of instrument #10912 is shown in Figure 2.

When even-numbered samples (N = 33) were used as unknowns to test the six calibrated instruments for accuracy of chlorophyll prediction, it was found that the average standard error of prediction (SEP) was 3.0 ppm (Table 2). This SEP was considered to be acceptable because duplicate chlorophyll analyses from the same grind, using the reference solvent extraction method, must differ by at least 2.5 ppm in order for the difference to be significant. A plot of predicted reflectance chlorophyll (instrument #10912) vs chlorophyll determined by solvent extraction is shown in Figure 3.

Development of a universal calibration equation. Manufacturers of NIR instruments will often supply universal calibration equations to customers for analysis of various components (e.g., protein in ground

TABLE 2

Chlorophyll Analysis Using the Modified Instalab 600[a]

Step	Wavelengths selected (nm)	Calibration (odds, N=33) R	SEE	Prediction (evens, N=33) R	SEP
1	674	0.852 (0.003)	7.8 (0.1)	0.858 (0.003)	8.6 (0.1)
2	696	0.976 (0.001)	3.3 (0.1)	0.981 (0.001)	3.3 (0.1)
3	2100	0.980 (0.001)	3.1 (0.1)	0.983 (0.001)	3.0 (0.1)

[a]Average of six instruments. Values in brackets represent standard deviation.

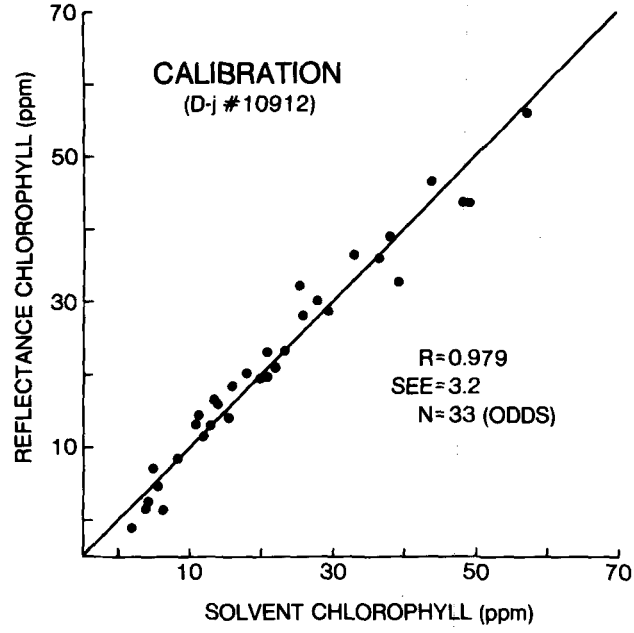

FIG. 2. Calibration plot for chlorophyll (seed basis, as is moisture) in ground rapeseed using instrument #10912.

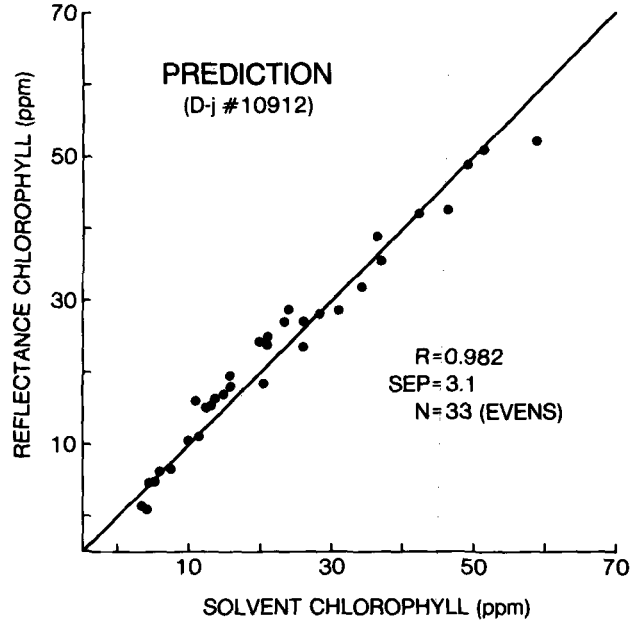

FIG. 3. Prediction of chlorophyll (seed basis, as is moisture) in ground rapeseed using instrument #10912.

wheat). The term "universal" implies that these equations can be used to calibrate any instrument, and one of their greatest advantages is that the universal calibration can be tested with as few as 20 samples and adjusted for bias to make the machine prediction agree with the reference analyses.

To develop a universal calibration equation for chlorophyll in ground rapeseed, reflectance data from all 66 samples (odds plus evens) were used to derive a more robust calibration for each of the six instruments. The regression constants for these equations are given in Table 3, and are in the form $y = a + b_0x_0 + b_1x_1 + b_3x_3$ where y is predicted chlorophyll; a is the intercept; b_0,b_1 and b_3 are the regression coefficients for filters F0 (674 nm), F1 (696 nm) and F3 (2100 nm); and x_0,x_1 and x_3 are the reflectance readings taken at those wavelengths. Because the calibration equations of all six instruments had similar coefficients, it was decided that the average of these six regression equations (Table 3) should be examined as a possible universal calibration equation.

To test the transferability of this universal calibration equation, reflectance data from the odd (N = 33) samples were used to correct each instrument for bias. To correct instrument #10912, for example, reflectance data for the odd samples were collected, and the universal calibration was then used to estimate the chlorophyll content of each. When these estimated chlorophyll values were regressed against the known solvent chlorophyll values, the resulting regression line had a slope of 1.067 and y-intercept of 1.844. Instrument #10912 was then adjusted for bias as follows:

$$\begin{aligned}\text{new intercept a} &= \text{universal a} \times \text{slope} + \text{intercept}\\ &= 1.6992 \times 1.067 + 1.844\\ &= 3.6567\\ \text{new } b_0 &= \text{universal } b_0 \times \text{slope}\\ &= 0.8926 \times 1.067\\ &= 0.9524\end{aligned}$$

$$\begin{aligned}\text{new } b_1 &= \text{universal } b_1 \times \text{slope}\\ &= -0.7867 \times 1.067\\ &= -0.8394\\ \text{new } b_3 &= \text{universal } b_3 \times \text{slope}\\ &= -0.1260 \times 1.067\\ &= -0.1344\end{aligned}$$

TABLE 3

Calibration Equation Regression Constants (N=66)

Instalab serial #	Intercept a	Regression coefficients b_0(674)	b_1(696)	b_3(2100)	R	SEE
10912	1.7013	0.9391	-0.7937	-0.1457	0.981	2.9
10914	1.8575	0.8785	-0.7789	-0.1241	0.982	2.9
10915	1.9294	0.8840	-0.7910	-0.1202	0.982	2.9
10916	1.0451	0.8854	-0.7896	-0.1174	0.982	2.9
10918	2.4878	0.8883	-0.7876	-0.1274	0.982	2.9
10920	1.1742	0.8806	-0.7794	-0.1209	0.982	2.9
Average[a]	1.6992	0.8926	-0.7867	-0.1260		

[a]Proposed universal calibration equation for chlorophyll in ground rapeseed.

TABLE 4

Bias Adjustment of the Universal Calibration Equation

Instalab serial #	Correction required for slope	intercept	Calibration[a] R	SEE	Prediction (evens, N=33) R	SEP
10912	1.067	1.844	0.975	3.4	0.985	2.8
10914	0.959	0.275	0.980	3.1	0.985	2.9
10915	0.959	0.352	0.979	3.1	0.985	2.8
10916	0.963	0.350	0.979	3.1	0.984	2.9
10918	0.971	0.442	0.980	3.1	0.984	2.9
10920	0.963	0.365	0.979	3.1	0.984	2.9
Average			0.979	3.2	0.984	2.9

[a]Adjusted for bias using odd (N=33) samples.

The slope and intercept corrections required for the bias adjustment of each machine are given in Table 4. It can also be seen from this table that after bias adjustment, the calibration equations for these six instruments had an average R of 0.979 and SEE of 3.2. Machine prediction of chlorophyll content agreed well with reference analyses when even-numbered samples (N=33) were used as unknowns (average SEP = 2.9 ppm), showing that the proposed calibration equation could be used with confidence. However, it must be pointed out that the 674 and 696 nm filters installed in all instruments were carefully matched for percent transmission, half peak band width, etc. It is not known if the equation for universal calibration presented in this paper could be transferred to instruments equipped with interference filters having different specifications.

One problem encountered in this study has been long term instrument drift caused by a gradual decrease in percent transmission of the 696 nm interference filter. While this drift has not affected the accuracy of chlorophyll determination, the frequent storage of new reference log readings (i.e., readings taken from the reference ceramic disk) required because of this drift has proven to be an inconvenience. We are presently investigating the stability of interference filters purchased from Micro Coatings (Westford, Massachusetts), and Spectrogon (Secaucus, New Jersey) in order to correct this problem.

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