A Comparison of High-Performance Liquid Chromatography and Spectrophotometry to Measure Chlorophyll in Canola Seed and Oil

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There are several methods available to measure chlorophyll in canola oil and seed, and these will not necessarily yield the same results and should not be used interchangeably. Total chlorophyll was determined for samples of canola seed and commercial canola oil by recognized spectrophotometric methods and by high-performance liquid chromatography (HPLC). The HPLC method, which summed all chlorophyll-related pigments detected, found approximately 1.4 times more total chlorophyll per sample than did the spectrophotometric methods. The spectrophotometric methods are calibrated with only chlorophyll a and underestimate other chlorophyll pigments, which have lower extinction coefficients and different absorption maxima. The HPLC method detects each pigment at its absorption maxima and applies the appropriate absorptivity factor. Care must be taken when comparing results obtained by different methods. There appears to be a need for a standardized method of chlorophyll pigment measurement by HPLC.

KEY WORDS: *Brassica*, canola oil, canola seed, chlorophyll, chlorophyll analysis, HPLC, pigments, spectrophotometer.

Chlorophyll measurement in canola seed and oil is important to monitor crop quality, particularly in years with early frost or for crops with late or uneven maturity. Several methods exist to measure total chlorophyll or individual chlorophyll components in canola seed and oil. These methods include the spectrophotometric AOCS Official Method AK 2-92 (1), which is applicable to the measurement of total seed chlorophyll in rapeseed, measured as chlorophyll a (chl a); the spectrophotometric AOCS Official Method Cc 13d-55 (2), which is applicable to the determination of total chlorophyll, measured as chl a, in refined vegetable oils (although this has generally been applied to canola oil at all stages of processing); and recently published (3-5) high-performance liquid chromatography (HPLC) methods for chlorophyll pigment measurement in canola oil. These methods rely on the separation of chlorophyll pigments on a reversed-phase HPLC column and detection with either an ultraviolet (uv)/visible or a fluorescence detector. These methods are calibrated with samples of each chlorophyll pigment detected. Different methods often will not yield the same results, so care must be taken when these results are compared. We have compared the results of chlorophyll analysis by two commonly used spectrophotometric methods for canola seed and oil samples, with results from HPLC by an unofficial but established method (4).

MATERIALS AND METHODS

Chlorophyll measurement in commercially extracted canola oil. Freshly extracted canola oil samples, including pressed, solvent-extracted and degummed oils, were obtained from a western Canadian canola crushing plant. Crude oil could not be obtained directly from the processor, and was prepared in the lab as a 50:50 mix of pressed and solvent-extracted oils. The fresh oil samples were placed in plastic bottles in a cooler and taken to the laboratory for immediate analysis. Samples were then stored for up to one month at room temperature in both light and dark, in a refrigerator and in a freezer.

HPLC technique. Oil was dissolved in acetone prior to analysis to give a solution of 25% oil. HPLC analysis was carried out according to the method of Endo *et al.* (4), except that the fluorescence detector was replaced with a photodiode array detector. The HPLC system consisted of two Waters (Waters Associates, Milford, MA) model 510 pumps, a Waters model 715 Ultra Wisp sample processor and a Waters model 994 programmable photodiode array detector. The column was stainless steel (220 mm × 4.6 mm) packed with O.D.S. 5 μm (Pierce Chemical Co., Rockford, IL). Each set of oil samples (fresh and after storage) was run on the HPLC by using a 50-μL injection volume and a run time of 30 min, which was sufficient to allow all of the chlorophyll derivatives to elute. The mobile phase was water/methanol/acetone (4:36:60) at a flow rate of 1.0 mL/min. The photodiode array detector was used to scan peaks to identify the chlorophyll pigments by their characteristic absorption maxima. Wavelengths used for quantitation were 642, 655, 662 and 667 nm. Chlorophyll pigments were identified from their absorption spectra and retention times by comparison with standards of known composition (4).

Chl a was purchased from Fluka Chemical Co. (Ronkonkoma, NY), and chlorophyll b (Chl b) was purchased from Sigma Chemical Co. (St. Louis, MO). Pheophytins a and b (phy a and phy b) were prepared from chl a and b, respectively, by reaction with HCl (6). Calibration curves were prepared for chl a, chl b, phy a and phy b. All curves were linear, with r^2 values ranging from 0.95–0.98. Endo *et al.* (4) had previously shown that these four compounds, plus pyropheophytin a, were the major pigments that occur in commercially-extracted canola oil.

Each set of samples included standards of chl b, chl a, b and phy a, and each day's run was quantitated by using the standards from the same run. Standards were not prepared for pheophorbide a, methylpheophorbide a or pyropheophytin a, so these were quantitated based on the phy a standard after multiplying by the ratio of the extinction coefficients (i.e., 1.24 for pheophorbide a, 1.33 for methylpheophorbide a and 1.10 for pyropheophytin a). Total chlorophyll for each oil sample was calculated as the sum of all chlorophyll derivatives detected.

Spectrophotometric method. The spectrophotometric method used was the AOCS Official Method Cc 13d-55 after recalibration with isooctane/ethanol instead of methylene chloride (2). Oil samples were dissolved in a 3:1 mixture of isooctane/ethanol to give a solution of 10% oil.

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Samples were filtered, and the absorbance was measured at 625, 665 and 705 nm in a 1-cm cuvette with a Varian (Palo Alto, CA) DMS 200 UV-Visible spectrophotometer. Two measurements were made on each sample, and the results were averaged.

Chlorophyll measurement in canola seeds. Canola seed was harvested at various stages of maturity throughout the ripening period to span a wide range of chlorophyll levels. These seed samples were analyzed by extracting the chlorophyll and measuring the absorbance with a Varian DMS 200 UV-Visible spectrophotometer, according to the AOCS Official Method AK 2-92 (1). One-gram samples of freeze-dried seed were weighed out and placed in stainless steel extraction tubes with three ball bearings and 30 mL of 3:1 isooctane/ethanol. Samples were shaken for one hour, filtered, dilutions were prepared if required, and the absorbance readings were measured. Three wavelengths were used (625.5, 665.5 and 705.5 nm) to measure the absorption peak for chlorophyll with corrections on either side. Two extractions and measurements were made on each sample, and the results were averaged. The absorbance readings were converted to chlorophyll levels by means of the following formula, which was developed from standards of known chlorophyll concentration:

$$\text{C.O.D.} = \text{Abs. (665.5 nm)} - [\text{Abs. (625.5 nm)} + \text{Abs. (705.5 nm)}]/2$$
$$\text{Chl (mg kg}^{-1}) = 390 * \text{C.O.D.} \quad [1]$$

where Abs. = absorbance; C.O.D. = corrected optical density.

To extract and prepare the samples for HPLC analysis, 2-g samples of freeze-dried seed were weighed out and placed in stainless-steel extraction tubes with three ball bearings and 30 mL of 3:1 isooctane/ethanol. Samples were shaken for 1 h and filtered, and the oil extract containing the chlorophyll was collected. High-chlorophyll samples were diluted if necessary. Samples were evaporated to dryness under nitrogen and resuspended in acetone. HPLC analysis was carried out as described for the oil samples, with an injection volume of between 10–50 μL, depending on the concentration of chlorophyll pigments in the sample.

RESULTS AND DISCUSSION

Chlorophyll measurement in commercially extracted canola oil. When total chlorophyll levels were measured by the spectrophotometric method for each oil sample, a small apparent decrease in total chlorophyll was observed in the pressed, solvent-extracted and crude oils during storage for one month (Figs. 1–3). The apparent decrease was greatest for the oils stored at room temperature in the light, followed by storage at room temperature in the dark, in the refrigerator and in the freezer, respectively. On the other hand, there was no apparent decrease in total chlorophyll during oil storage under any of the conditions tested in the degummed oils (Fig. 4).

Previous studies have never indicated a decrease in total chlorophyll during oil storage. In another paper (7), we showed that during oil storage chl b was converted to phy b, and chl a was converted to phy a and pyropheophytin a. This conversion occurred most quickly in oils stored at room temperature in the light, followed by storage at room temperature in the dark and in refrigerated storage, respectively. Only minor changes occurred in oil samples that were frozen.

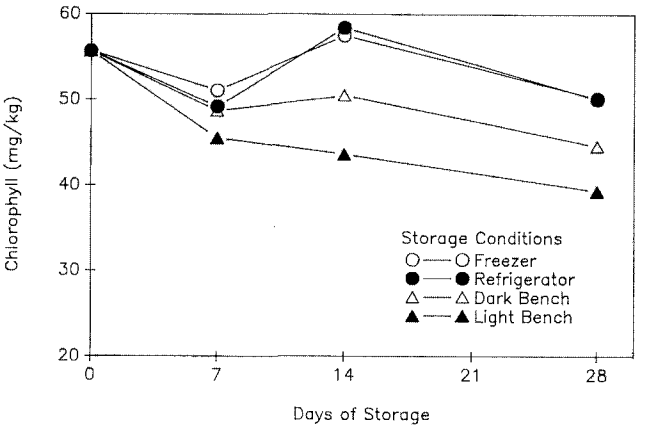

FIG. 1. Total chlorophyll in pressed oil during storage as measured by spectrophotometry.

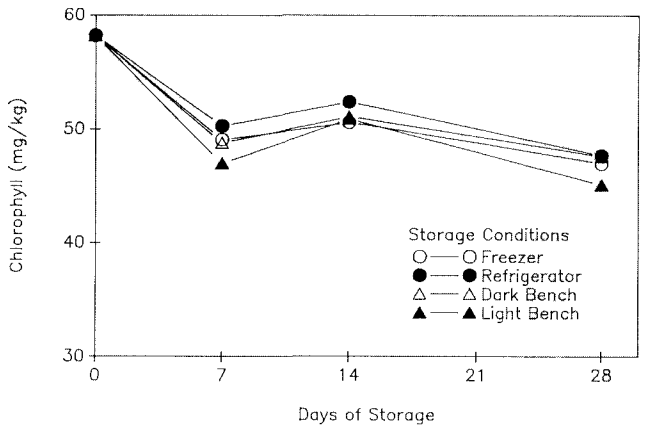

FIG. 2. Total chlorophyll in solvent-extracted oil during storage as measured by spectrophotometry.

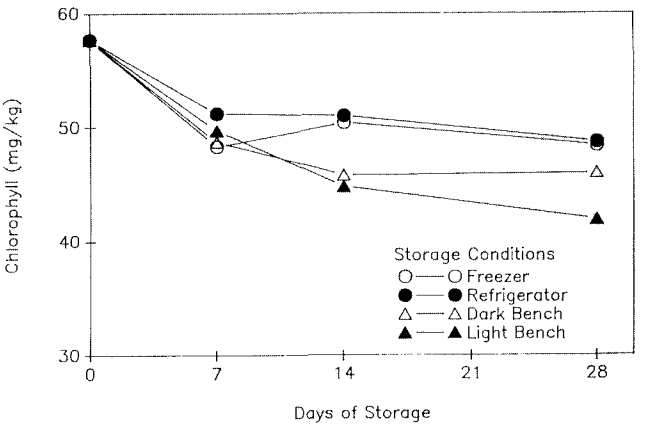

FIG. 3. Total chlorophyll in crude oil during storage as measured by spectrophotometry.

Therefore, the apparent decrease in total chlorophyll during storage, as measured by the spectrophotometric method, was not a real decrease. During storage, chlorophylls were converted to pheophytins and some pyropheophytins. The spectrophotometric method of chlorophyll

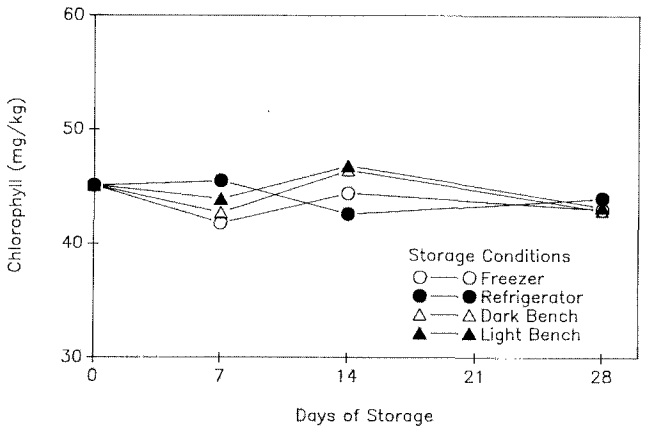

FIG. 4. Total chlorophyll in degummed oil during storage as measured by spectrophotometry.

measurement is calibrated with only chl a, which has a much higher extinction coefficient (at 665 nm) than phy a or any of the other chlorophyll derivatives (Table 1). Thus, pheophytins and pyropheophytins in the stored oil samples, although present at the same concentration as the original chlorophylls, will produce a lower absorbance reading on the spectrophotometer, leading to an underestimate of total chlorophyll. In the pressed, solvent-extracted and crude oils, the conversion of chl a to phy a and pyropheophytin a was responsible for the apparent decrease in total chlorophyll observed. The degummed oil contained mainly pheophytins and pyropheophytins to begin with, so little conversion was possible, and there was no decrease in absorbance.

Suzuki and Nishioka (5) also discussed this apparent discrepancy between pigment concentrations when measured by the AOCS spectrophotometric method Cc 13d-55 and by an HPLC method. They detected concentrations of pheophytins and pyropheophytins that were 1.4 times higher by HPLC than by the spectrophotometric method. They also stated that the calibration of the spectrophotometric method with chl a accounted for this observation.

In our study, total chlorophyll, as determined by HPLC and calculated as the sum of all chlorophyll derivatives detected, did not agree with total chlorophyll as measured by the spectrophtometric method. There was a strong positive correlation between total chlorophyll levels determined by HPLC and spectrophotometry ($r = 0.73$, $P > |r| = 0.0001$). The mean ratio of HPLC chlorophyll/spectrophotometer chlorophyll was 1.40 with a standard deviation of 0.27. Therefore, the HPLC method detected, on average, 1.4 times more total chlorophyll per sample than did the spectrophotometric method. However, this value varied a great deal between samples.

Chlorophyll measurement in canola seeds. The relationship between total chlorophyll as measured by HPLC and calculated as the sum of all chlorophyll derivatives detected in a seed sample, and total chlorophyll, measured by the AOCS spectrophotometric method AK 2-92 (1), was compared for seed samples ranging from green to fully ripe, to cover a wide range of chlorophyll levels.

The HPLC method detected, on average, 1.4 times more total chlorophyll than the spectrophotometric method. This value of 1.4 was identical to that found for the commercially-extracted oil samples in the previous section. It also agrees with the results of Suzuki and Nishioka (5). There was a high positive correlation (+0.93) between total chlorophyll as measured by HPLC and spectrophotometry, significant at the $P = .0001$ level.

The ratio between the HPLC measurement of total chlorophyll pigments and the spectrophotometric readings varied between 0.3–3.0 for individual samples. Over the entire study, the ratio of HPLC/spectrophotometer chlorophyll was 1.37 with a standard deviation of 0.43. Any samples that contained less than 3.0 mg $\cdot$ kg^{-1} total chlorophyll, as measured by the spectrophotometric method, were not included in the calculations. The spectrophotometric method only detects chlorophyll to an accuracy of within 3 mg $\cdot$ kg^{-1}; therefore, samples containing lower levels of chlorophyll are below the limits of accurate detection, and the ratio for these low pigment levels could be abnormally high or low by chance alone. Once again, the discrepancy in total chlorophyll, as measured by HPLC and spectrophotometry, is explained by the calibration of the spectrophotometric method in which only chl a is used, leading to an underestimation of any other pigments present.

These results illustrate a potential problem in chlorophyll analysis studies. For any given study, the same method of chlorophyll measurement must be used throughout the entire study, as results from different methods cannot be directly compared. Also, the results of separate studies, which have been carried out with different methods of chlorophyll measurement, should not be directly compared. The spectrophotometric and HPLC methods of chlorophyll measurement were not designed for the same use. The spectrophotometric method gives a relatively rapid measurement of total chlorophyll and was designed to compare samples of the same type. The

TABLE 1

Absorption Characteristics of Chlorophylls and Related Pigments

Pigment	Maximum λ	E[a]	Maximum λ	E[a]	Reference
Pheophorbide a	409	119200	667	55200	8
Methylpheophorbide a	408.5	122500	667	59200	9
Chlorophyll b	455	131000	645	47100	10
Chlorophyll a	430	94700	663	75000	10
Pheophytin b	434.5	145000	654	27800	8
Pheophytin a	409	101800	666	44500	11
Pyropheophytin a	409	102400	667	49000	9

[a]E, Absorbtivity (molar extinction coefficient) in acetone (except methylpheophorbide a in ether).

more diverse the samples that are being compared, the greater the degree of error that may be introduced. For example, a high-quality seed sample that contained 15 mg · kg^{-1} chlorophyll a would produce a higher absorbance reading than a damaged seed sample that contained 15 mg · kg^{-1} phy a.

The HPLC method, on the other hand, can accurately determine the levels of each chlorophyll pigment in a sample. Therefore, it can be used to make comparisons between different types of samples. It is, however, much more time-consuming and expensive than the spectrophotometric method if one is only interested in total chlorophyll. As more laboratories purchase HPLC equipment, a standardized HPLC method for chlorophyll determination in canola seed and oil would be useful.

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