# Characterization of Chlorophyll Pigments Present in Canola Seed, Meal and Oil[1]

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**Chlorophyll pigments present in canola seed, meal and crude and degummed oils were analyzed by high-performance liquid chromatography (HPLC) with a fluorescence detector. Chlorophylls a and b, low levels of pheophytin a, and occasionally traces of pheophorbide and its methyl ester were present in canola seed. Meals and oils contained magnesium-deficient chlorophyll pigments such as pheophorbide a, methylpheophorbide a, pheophytins a and b, and pyropheophytins a and b but not chlorophyll a or b. The amounts of chlorophyll pigments were oil > seed >> meal. Both crude and degummed oils contained pheophytin a and pyropheophytin a as main components, but the ratio of pyropheophytin a to pheophytin a was markedly higher in degummed oils. No pheophorbides were detected in degummed oils. These results suggest that oil processing steps such as extraction and degumming affect the composition of chlorophyll pigments.**

KEY WORDS: Canola, chlorophyll, HPLC, meal, oil, pheophorbide, pheophytin, pigment, pyropheophytin, seed.

Chlorophyll pigments present in canola and other oilseeds are important quality factors because they impart undesirable color to vegetable oils and can promote oxidation in the presence of light (1–3) and inhibit hydrogenation (4,5). Moreover, phytol-deficient chlorophylls such as chlorophyllides and pheophorbides may present a nutritional concern because of their photo-toxicity, which may result in photosensitive dermatitis (6,7). A bleaching step is necessary during oil processing to remove chlorophyll-related pigments and other color bodies. Its efficiency has been suggested to depend on levels and varieties of chlorophyll pigments (8).

In previous studies, we have applied spectrophotometry (9), fluorophotometry (10,11), near-infrared reflectance spectrophotometry (12) and high-performance liquid chromatography (HPLC) with photodiode array detection (13) to the investigation of chlorophyll pigments in oilseeds and vegetable oils. These studies showed that chlorophylls a and b, which exist as the main components in oilseeds, were not always present in vegetable oils. Vegetable oils such as canola oil contained pheophytins a and b as the main chlorophyll pigments instead of chlorophylls a and b while unknown chlorophyll pigments were also detected (13).

In this study we utilized HPLC equipped with photodiode array and fluorescence detectors to characterize and quantitate chlorophyll pigments present in canola seeds, meals and crude and degummed oils to determine changes that take place in chlorophyll pigments during oil processing.

## MATERIALS AND METHODS

*Materials.* Chlorophylls a and b were purchased from Sigma Chemical Co. (St. Louis, MO). Pheophytin a and b and pheophorbide a and b were prepared from chlorophyll a and b by reaction with HCl (1). Pyropheophytins a and b were prepared by the method described by Kenner *et al.* (14). Methyl pheophorbide a was prepared by reaction of pheophorbide a with hydrochloric acid and methanol (15). The concentrations of standard solutions of chlorophyll pigments were determined spectrophotometrically by using specific absorbancies shown in Table 1.

Canola seeds, corresponding meals and crude and degummed oils prepared from the seeds were obtained from Canadian canola crushing plants. Samples were chosen with levels of total chlorophyll covering the normal range found in Canadian canola. The levels included two samples with about 10 mg/kg chlorophyll, typical of processing *B. campestris* seed types, three samples with levels near 20 mg/kg, typical of those found when processing *B. napus* seed types, and a sample with more than 30 mg/kg, well above the industry standard for top quality.

*Extraction of chlorophyll pigments.* To obtain chlorophyll pigments from canola seeds and meals, 2 g of ground seed (coffee mill) or meal were extracted with 30 mL of isooctane/ethanol (3:1) in a ball mill (16). This method gives complete extraction of chlorophyll pigments from canola seed with no degradation (17). Extracts were evaporated with a rotary evaporator and made up to 5 mL with acetone prior to HPLC analysis. Crude and degummed oils were dissolved in acetone to give a solution of 25% oil.

*HPLC analysis.* HPLC was carried out on a system consisting of 2 Waters (Milford, MA) 510 pumps, a Waters 715 Ultra WISP sample processor, a Hewlett-Packard HP 1046 Programmable fluorescence detector (Palo Alto, CA). The column was a stainless steel cartridge (220 mm × 4.6 mm) packed with ODS 5 $\mu$M (Pierce Chemical Co., Rockford, IL). The mobile phase was 10% water-methanol (0.4 mL/min) and acetone (0.6 mL/min). Chlorophyll pigments were detected with two different fluorescence programs (1:Ex405-EM670 nm, 2:Ex430-Em655 nm). A Waters 994 programmable photodiode array detector was attached in series with the fluorescence detector and was used to scan peaks in order to assist in identification of chlorophyll components by their characteristic absorption maxima. Chlorophyll pigments were identified by their absorption spectra and retention times by comparison with known compounds.

Calibration curves were prepared with standard solution of chlorophylls a and b, and magnesium-deficient compounds of chlorophyll a, such as pheophorbide a, methylpheophorbide a, pheophytin a and pyropheophytin a, by relating the peak area from fluorescence chromatograms to the spectrophotometrically calibrated concentrations.

## RESULTS AND DISCUSSION

The HPLC system provided good separation on a solution of 9 chlorophyll-based pigments (chlorophyll a and b, pheophorbide a and b, methylpheophorbide a, pheophytin a and b, and pyropheophytin a and b) although peaks of pheophytin a and pyropheophytin b overlapped

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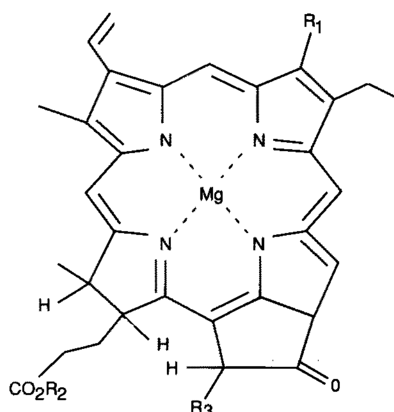
TABLE 1

**Structures and Spectral Properties of Chlorophylls a and b, and Their Derivatives**




| Name | Abbrev. | X | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|---|
| Chlorophyll a | Chl a | Mg | $CH_3$ | $C_{20}H_{39}$[a] | $CO_2CH_3$ |
| Pheophorbide a | Pho a | $H_2$ | $CH_3$ | H | $CO_2CH_3$ |
| Methylpheophorbide a | MePho a | $H_2$ | $CH_3$ | $CH_3$ | |
| Pheophytin a | Phy a | $H_2$ | $CH_3$ | $C_{20}H_{39}$ | $CO_2CH_3$ |
| Pyropheophytin a | Pyr a | $H_2$ | $CH_3$ | $C_{20}H_{39}$ | H |
| Chlorophyll b | Chl b | Mg | CHO | $C_{20}H_{39}$ | $CO_2CH_3$ |
| Pheophorbide b | Pho b | $H_2$ | CHO | H | $CO_2CH_3$ |
| Pheophytin b | Phy b | $H_2$ | CHO | $C_{20}H_{39}$ | $CO_2CH_3$ |
| Pyropheophytin b | Pyr b | $H_2$ | CHO | $C_{20}H_{39}$ | H |

| | E = Absorbtivity (molar extinction coefficient)[b] | | | | |
|---|---|---|---|---|---|
| | Max. | E | Max. | E | Ref. |
| Chlorophyll a | 430 | 94700 | 663 | 75000 | 18 |
| Pheophorbide a | 409 | 119200 | 667 | 55200 | 19 |
| Methylpheophorbide a | 408.5 | 122500 | 667 | 59200 | 15 |
| Pheophytin a | 409 | 101800 | 666 | 44500 | 20 |
| Pyropheophytin a | 409 | 102400 | 667 | 49000 | 21 |
| Chlorophyll b | 455 | 131000 | 645 | 47100 | 18 |
| Pheophorbide b | 439 | 154000 | 653 | 39800 | 18 |
| Pheophytin b | 434.5 | 145000 | 654 | 27800 | 19 |

[a]Phytyl Group; [b]In acetone solution (MePho in ether solution).

(Fig. 1). All chlorophyll pigments gave good responses on the fluorescence detector, even though the HP detector does not operate at its most optimum sensitivity at these wavelengths. Chlorophyll b was relatively insensitive at the fluorescence program Ex405-Em670 nm (Table 2).

Chlorophylls a and b were the main chlorophyll pigments in canola seeds, with pheophytin a present as minor component. Traces of pheophorbide a and its methyl ester (methylpheophorbide a) were also observed in some canola seeds (Fig 1, Table 3). Methylpheophorbide a has been reported as a chlorophyll a degradation product in chlorella (22), but until now it has not been reported in canola.

Chlorophyll a levels were about three times as high as chlorophyll b, while pheophytin a, pheophorbide a and methylpheophorbide a levels were low. These observations were consistent with previous results (13).

Canola meals (Fig. 1) contained low levels of pheophorbide a, methylpheophorbide a, pheophytins b and a (containing traces of pyropheophytin b), and pyropheophytin a, but not chlorophylls a and b. Moreover, pheophytins a′ and b′, possibly chiral isomers at position C-10 of pheophytins a and b, were also observed. Pheophytin a and pyropheophytin a were the main components in canola meals while pheophorbide a, methylpheophorbide a and pheophytin b were present as minor components (Table 3). The total levels of chlorophyll pigments in meals (1.2–5.2 mg/kg) were about 1/10 of that in the corresponding seeds (11 to 33 mg/kg).

Although chlorophylls a and b were not detected in crude and degummed oils (Fig. 1), the presence of pheophytins b and a (containing traces of pyropheophytin b), pyropheophytin a, and probably pheophytins a′ and b′ were confirmed by fluorescence detection. Traces of methylpheophorbide a were also observed in crude and degummed oils. Significant differences were observed between crude and degummed oils. With fluorescence detection, pheophorbide a was observed in crude oils but not in degummed oils. Although the main chlorophyll pigments in both crude and degummed oils were pheophytin a and pyropheophytin a, the ratio of pyropheophytin a and pheophytin a in degummed oils (2.6) was much higher than that in crude oils (0.4) (Table 3). There

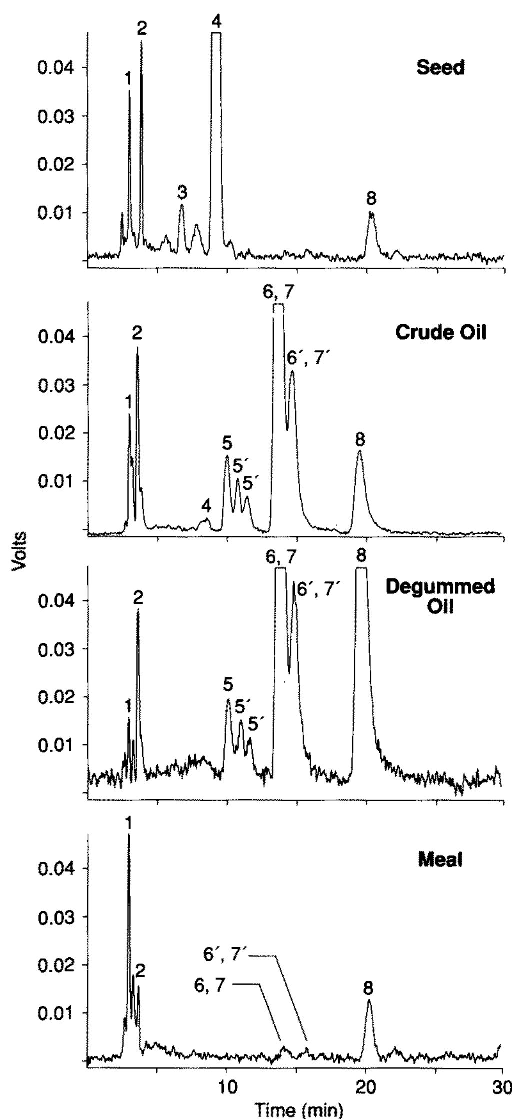



**FIG. 1. Chromatograms of chlorophyll pigments from canola seed, crude oil, degummed oil and meal; 1 = pheophorbide a; 2 = methylpheophorbide a; 3 = chlorophyll b; 4 = chlorophyll a; 5 = pheophytin b; 6 = pheophytin a; 7 = pyropheophytin b; 8 = pyropheophytin a. Primed numbers indicate epimers.**

was no significant difference in the total level of chlorophyll pigments between crude and degummed oils.

From these results, it is possible to follow the changes in chlorophyll pigments that take place during oil processing from seeds to degummed oils (Fig. 2). Because pheophytins were found in meals and oils, but not chlorophylls, it appears that most of the chlorophylls a and b, the predominant pigments in canola seed, lose magnesium and are converted to their respective pheophytins during the extraction steps (pressing and solvent extraction). In some cases the extraction process removed the phytol from pheophytins, converting them to pheophorbides and their methyl esters. Usually, only pheophorbide a and methylpheophorbide a, but not pheophorbide b, were formed because removal of phytol from pheophytin b needs more severe reactive conditions than for pheophytin a (21). Pyropheophytins are the main chlorophyll pigments in degummed oils while pheophytins are the main components in crude oils. There was no difference in the pattern of chlorophyll pigments extracted between expelled or pressed oils and solvent-extracted oils. The methoxy carboxyl group of pheophytins can be removed to form pyropheophytins during heat treatment in the presence of phosphoric and either citric or malic acids. The degumming process also removes pheophorbide a from oil, probably due to washing.

TABLE 2

**Calibration Parameters for Chlorophyll Compounds**[a]

| Compound | Slope (ng/volt-min) | Intercept (ng) | C.V. (%) |
|---|---|---|---|
| Chlorophyll a | 138.7 | 2.5 | 2.6 |
| Chlorophyll b | 1445.3 | −0.9 | 4.2 |
| Pheophytin a | 99.7 | 4.0 | 5.8 |
| Pheophytin b | 106.9 | 2.6 | 1.5 |
| Pyropheophytin a | 127.9 | 0.5 | 3.3 |
| Pheophorbide a | 65.9 | 16.5 | 2.9 |
| Methylpheophorbide a | 108.2 | 2.9 | 2.7 |

[a]For HPLC conditions as described in text with fluorescence program of excitation 405 nm, emission 670 nm, except pheophytin b which was detected at a fluorescence program of excitation 430 nm, emission 655 nm.

The major chlorophyll pigments (about 90%) in oils were a-type (pheopohytin a and pyropheophytin a), although the ratio of chlorophyll a:b was about 3:1 in canola seeds. Possibly the b-type pigments are not as susceptible to extraction from seeds with nonpolar solvent, such as *n*-hexane, although the proportion of the b-type pigments found in meals was similar to that in oils (it is possible that some of the pheophytin a found in meals was actually pyropheophytin b). Due to their higher polarity, b-type pigments are also more washable in comparison with a-type (9).

Many researchers have investigated chlorophyll pigments in oilseeds and oils (17,23–26). Fraser and Frankel (24), Aitzetmuller (25) and Davies *et al.* (26) investigated chlorophyll pigments present in vegetable oils by using HPLC and found pyropheophytins in oils. We also suggested the presence of pyropheophytins and pheophorbides in canola and soybean oils but could not identify them in a previous paper (13).

In this paper, we have applied HPLC combined with fluorescence and photodiode array detection for canola seeds, meals and oils, and confirmed the presence of pyropheophytin a, pheophorbide a and methyl pheophorbide a as well as pheophytins a and b in meals and oils. Moreover, we have almost completely succeeded in determining the chlorophyll pigments present in canola seeds, meals and oils for the first time.

From these results, it is clearly demonstrated that oil processing, such as extraction and degumming, affects the composition of chlorophyll pigments in oils. This HPLC method makes it possible to estimate the chlorophyll pigments present in oils, especially the ratio of pyropheophytin a and pheophytin a, and the presence of pheophorbide a in oils. We believe that this HPLC method is amenable for use in quality control of vegetable oils, oilseeds and meals.

TABLE 3

**Chlorophyll Pigments in Samples of Canola Seed, Meal and Oil**[a]

| Plant | Process step | Chlorophyll Pigments (mg/kg) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Chl a | Chl b | Phy a | Phy b | Pho a | MePho a | Pyr a | Total |
| A | Seed | 13.3 | 6.6 | <0.1 | | | | | 19.9 |
| | Meal | | | 1.3 | 0.2 | 0.4 | | 1.5 | 3.3 |
| | Crude | | | 35.2 | 5.8 | 1.0 | 2.0 | 6.7 | 50.7 |
| A | Seed | 17.6 | 5.8 | 0.3 | | 0.1 | 0.1 | | 23.9 |
| | Meal | | | 0.9 | 0.1 | 0.4 | 0.1 | 0.6 | 2.1 |
| | Crude | | | 22.0 | 2.0 | 0.6 | 0.8 | 3.2 | 28.6 |
| A | Seed | 8.4 | 2.5 | 0.4 | | <0.1 | | | 11.3 |
| | Meal | | | 0.7 | 0.1 | <0.1 | | 0.4 | 1.2 |
| | Crude | | | 13.4 | 5.4 | 0.3 | 0.4 | 1.2 | 20.7 |
| B | Seed | 9.0 | 2.5 | <0.1 | | | | | 11.5 |
| | Meal | | | 0.9 | 0.5 | 0.3 | | 0.5 | 2.1 |
| | Degummed | | | 9.0 | 0.2 | | 0.3 | 6.1 | 15.6 |
| C | Seed | 18.6 | 4.7 | 0.8 | | <0.1 | | | 24.1 |
| | Meal | | | 2.4 | 0.4 | 0.2 | 0.1 | 2.1 | 5.3 |
| | Degummed | | | 6.8 | 2.4 | | 0.3 | 23.6 | 33.1 |
| D | Seed | 23.7 | 8.3 | 1.0 | | 0.1 | 0.1 | | 33.2 |
| | Meal | | | 0.9 | 0.4 | 0.2 | | 0.7 | 2.1 |
| | Degummed | | | 8.8 | 3.0 | | 0.5 | 30.9 | 43.2 |

[a]Chl a = Chlorophyll a; Chl b = Chlorophyll b; Phy a = Pheophytin a (and pyropheophytin b); Phy b = Pheophytin b; Pho a = Pheophorbide a; MePho a = Methylpheophorbide a; Pyr a = Pyropheophytin a.

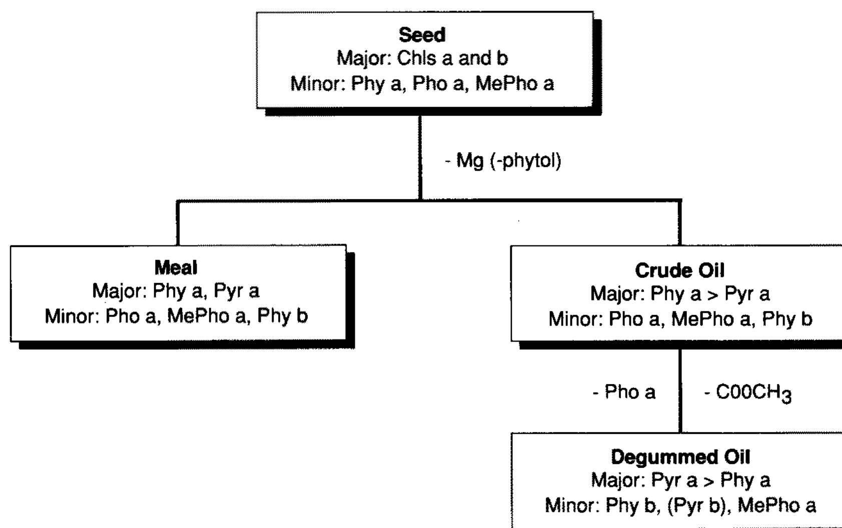



**FIG. 2. Changes in chlorophyll pigments during oil processing.**

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