Determination of Chlorophyll Pigments in Crude and Degummed Canola Oils by HPLC and Spectrophotometry[1]

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Chlorophyll pigments in crude and degummed canola oils were analyzed by spectrophotometry using a modified AOCS Method and by reversed phase HPLC. HPLC showed that crude canola oils contained very little *chlorophyll a* or *b*, these pigments having been converted to pheophytins and other pigments with similar spectral properties. The ratio of *chlorophyll a:b* in the seed was found to be about 3:1 while the ratio of *pheophytin a:b* in the oil was about 9:1. As the AOCS Method for determining oil chlorophyll was calibrated for pure chlorophyll, the use of this method on crude canola oil results in a significant error. Recalibration of the spectrophotometric procedure with pheophytin gave better agreement with the HPLC method.

The level of chlorophyll-related pigments in canola and spring-grown rapeseed is an important factor for oil quality (1-3). Chlorophyll pigments not only impart an undesirable color to vegetable oils but they have been shown to impair hydrogenation (4) and to promote oxidation in the presence of light (5-8) although they may be antioxidants in dark conditions (9,10). Removal of chlorophyll pigments from canola oil by conventional processing techniques is difficult and may require additional bleaching steps compared with other oils (11).

In 1955, in recognition of a similar problem in soybean oil, the oil color committee of the AOCS developed a method for the determination of chlorophyll in refined and bleached vegetable oils (12-15). A variation of this method (16) was adopted by Canada as a part of its National Standard for crude, degummed and refined canola oils (17).

Although the AOCS method was originally calibrated for the determination of pure *chlorophyll a*, various authors have since demonstrated that chlorophyll is almost completely decomposed during conventional extraction procedures and that crude canola and rapeseed oils contain primarily pheophytin and other chlorophyll-related products (2,19-22). High performance liquid chromatography (HPLC) studies of soybean (23) and olive oil (24) have shown similar results. The specific absorbances of pheophytins are different from the specific absorbances of chlorophylls at the analytical wavelengths used in the AOCS method (Table 1). This has resulted in inaccurate quantitation of the amount of pigment present and has led to subsequent difficulties in relating the true chlorophyll content of canola seed with the amount of pigments found in the crude oil extracted from that seed.

This paper describes an HPLC approach for determination of chlorophyll pigments in crude canola oil. A comparison is made between the HPLC procedure and the AOCS method. A modification of the AOCS method is proposed which results in closer agreement for estimation of pigments by these two methods.

MATERIALS AND METHODS

Materials. *Chlorophylls a* and *b* were obtained from the Aldrich Chemical Company, or from Fluka Co. From Fluka β-apo-8′-carotenal was obtained. Pheophytins were prepared from chlorophylls by reaction with HCl(25). The concentrations of standard solutions of chlorophyll and pheophytin were determined spectrophotometrically using specific absorbances given by the AOAC (26). The red:blue absorption ratios and positions of absorption maxima agreed with the literature values (27) for all standards.

Heptane:ethanol extracts from canola seeds were obtained by extraction of 2 g ground seed with 30 ml heptane:ethanol (3:1) using a ball mill (28). Crude canola oils were obtained from Canadian canola crushing plants. Crude oils were dissolved in heptane:ethanol (3:1) to give a solution of about 25% oil prior to HPLC.

HPLC-grade solvents, isooctane, methanol, acetonitrile and dichloromethane were obtained from Fisher Scientific Co. HPLC grade water was prepared on a Barnstread Nanopur System. Heptane, ethanol, diethylether and methyl-tertiarybutylether were ACS grade or better.

Chromatography. Chromatography was carried out on a system consisting of 3 Waters model 510 pumps, a Waters model 712 WISP auto-injection system, and a Waters model 490 programmable multi-wavelength UV/Vis detector. As the Waters model 490 detector has a wavelength maximum of 600 nM, chlorophyll pigments were determined at their absorption maxima between 408 nM and 450 nM using the maxplot facility at 408 nM, 430 nM and 450 nM.

HPLC columns were (a) stainless steel cartridge 220 mm × 4.6 mm i.d. packed with ODS 5μM (Pierce Chemical Co.) or (b) a radial compression cartridge, 100 mm × 8 mm, packed with 4μM ODS (Waters). Mobile phases were: A — acetronitrile; B —isooctane/dichloromethane (1:1); C — water/methanol (1:9). The gradi-

TABLE 1

Spectral characteristics of chlorophyll and pheophytin[a]

	Maximum Wavelength (nm)		Specific Absorption Coefficient	
Pigment	Red	Blue	Red	Blue
Chlorophyll a	662	430	100	130
Chlorophyll b	648	453	55	107
Pheophytin a	667	408	60	135
Pheophytin b	655	434	40	220

[a]Reference (27), All measurements for diethylether solutions.

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ents for Column A were: flow 1 ml per min from 100% C at 0 min linearly to 45% C, 40% A and 15% B at 18 min; linearly to 80% A, 15% B, and 5% C at 28 min. The column was cleaned between runs by increasing to 100% B followed by 100% A, and 100% C. For the Radial Pak column, a similar system was used but the total time was decreased to 20 min and the flow was 3 ml per min. Integration, data processing and chromatography system control were accomplished using Waters 840 System software operating on a DEC Pro 350 computer.

Spectrophotometry. Spectrophotometric measurements were made on a Bausch and Lomb Spectronic 1001 split beam spectrophotometer and spectra were obtained from a Beckman ACTA M-VI double beam spectrophotometer and a Beckman DU-7 Spectrophotometer.

RESULTS AND DISCUSSION

The HPLC approach described by Khachik, Beecher and Whitaker (29) for simultaneous measurement of chlorophyll and carotenoid pigments in green vegetable extracts was modified for estimation of chlorophyll pigments in canola oil. Solvents of lower polarity than specified by Khachik *et al.* were required to completely elute the pheophytins. This was likely due to differences in the columns or to a matrix effect caused by the relatively large amount of lipid in our samples. A good separation of *chlorophylls a* and *b* and their respective pheophytins was possible although the internal standard (also recommended by Khachik and Beecher (30)) eluted close to the *chlorophyll b* peak (Figure 1). The radial pak column was considerably faster and gave the same separation except for the pheophytin a peak which was not well separated from the later eluting peaks.

The source of chlorophyll and the method used in the preparation of standard reference solutions were found to be extremely important. In some solutions, although no spectral anomalies were observed, HPLC showed multiple peaks indicating allomerization of the chlorophyll (Figure 2) (31). Allomers sometimes were present in the chlorophyll as purchased and pure chlorophyll dissolved in ether gradually allomerized. In order to minimize allomerization problems, the chlorophyll standard solutions were analyzed by HPLC as soon as possible, even before spectrophotometric determination of their contents. Allomerization was not a problem in seed extracts or oil samples.

The predominant chlorophyll pigments found in heptane:ethanol extracts from canola seeds, were *chlorophyll a* and *chlorophyll b* (Figure 3). Small amounts of *pheophytin a* were also found in agreement with results from fluorescence analysis (32). Analysis of a large number of samples with varying levels of chlorophyll showed that the ratio of *chlorophyll a* to *chlorophyll b* was about 3:1 (Figure 4). This was significantly different from the ratio of 9:1 or greater noted by several other workers based on purely spectrophotometric measurements on seeds (31) or commercially produced oils (19). The differences may be due either to errors in the spectrophotometric methods caused by the matrix of components present or to the different sources and different moisture contents of the seeds. The ratio of *chlorophyll a:b* in the present study was similar to the ratio found by Usuki and Endo (31).

Analysis of canola oil samples (Figures 5) showed that the major chlorophyll species present was *pheophytin a.* Only small amounts of *pheophytin b* were found, in agreement with the spectrophotometric stud-

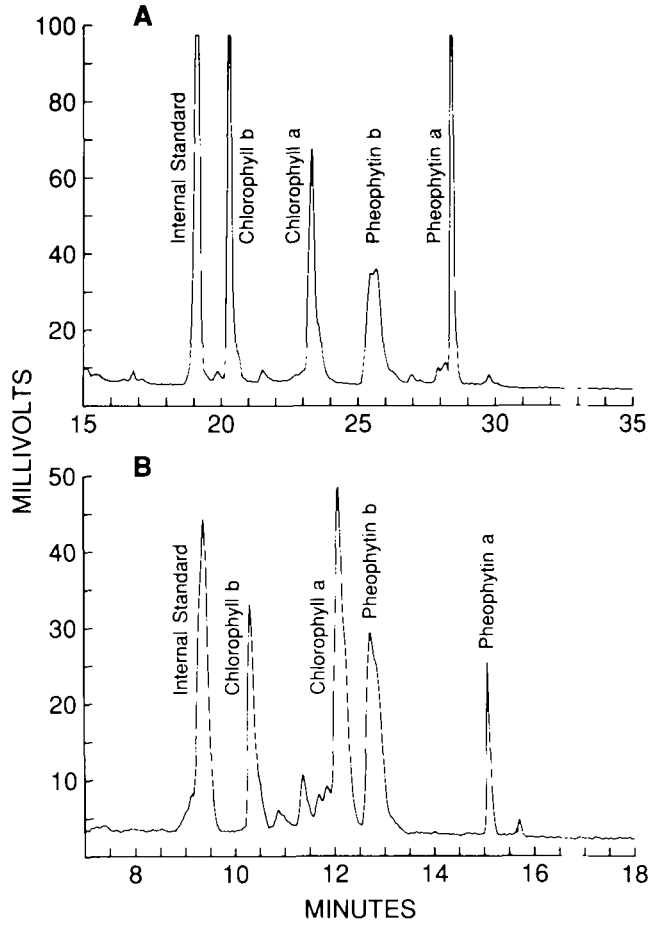

FIG. 1. Separation of chlorophylls and pheophytin standards on a) steel cartridge and b) radial pac ODS columns. Chromatographic conditions as described in text.

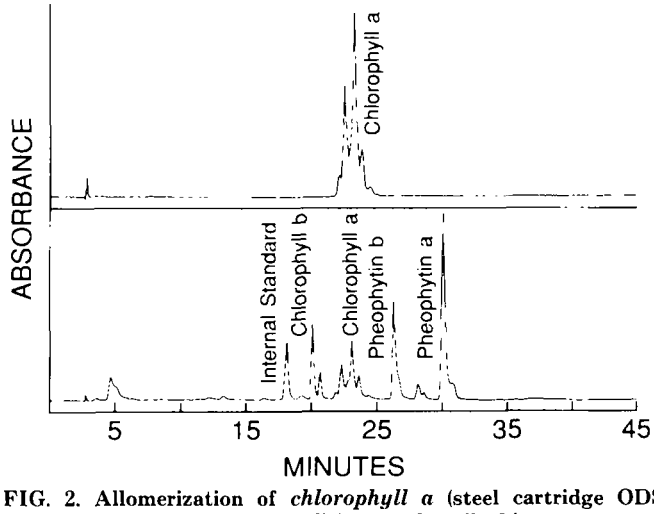

FIG. 2. Allomerization of *chlorophyll a* (steel cartridge ODS column). Chromatographic conditions as described in text.

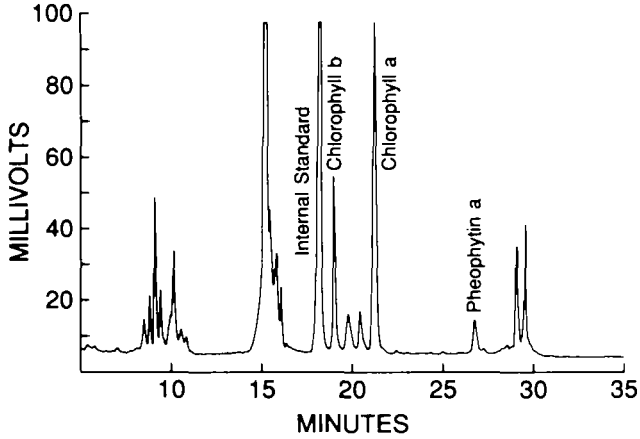

FIG. 3. Chlorophyll pigments in canola seed extract (steel cartridge ODS column). Chromatographic conditions as described in text.

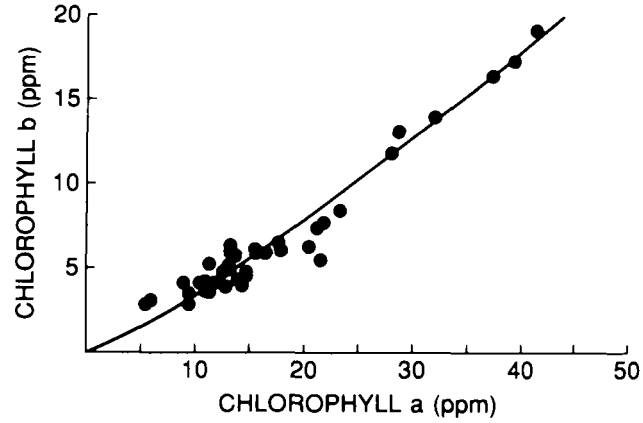

FIG. 4. Relationship between *chlorophyll a* and *chlorophyll b* in heptane:ethanol extracts of canola seed.

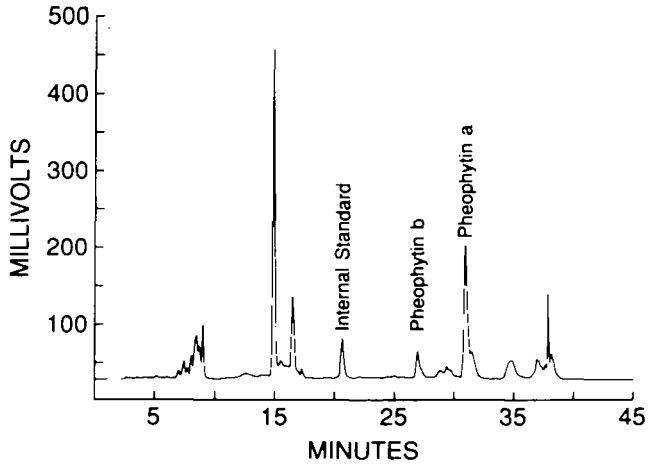

FIG. 5. HPLC analysis of chlorophyll pigments in crude canola oil (steel cartridge ODS column). Chromatographic conditions as described in text.

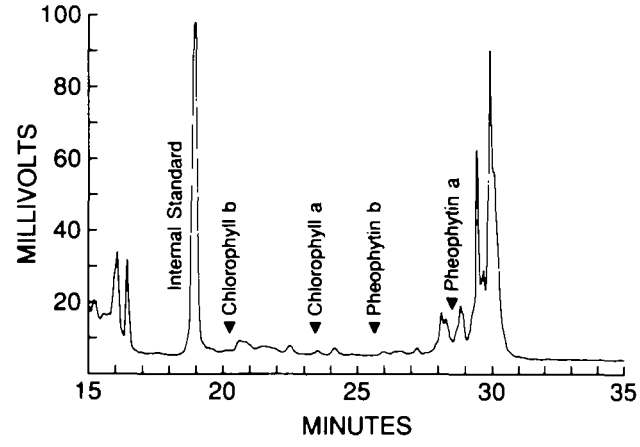

FIG. 6. HPLC analysis of chlorophyll pigments in crude soybean oil (steel cartridge ODS column). Chromatographic conditions as described in text.

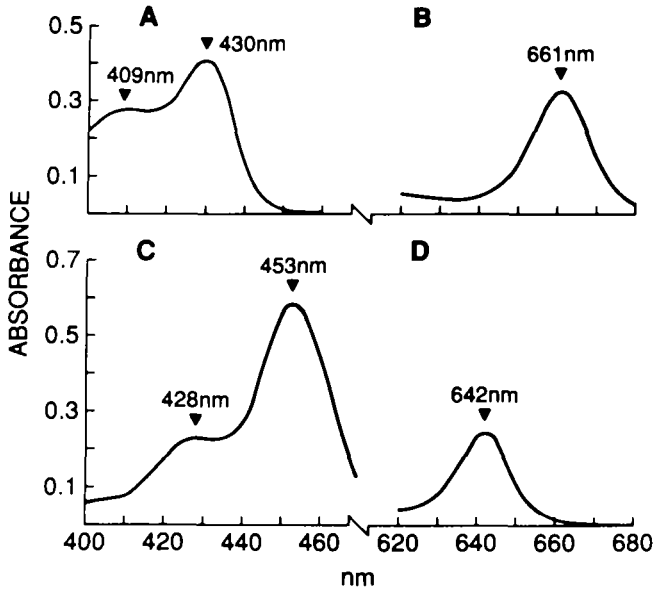

FIG. 7. Absorption spectrum of *chlorophyll a* (a,b) and *chlorophyll b* (c,d) fractions collected from HPLC analysis of heptane:ethanol extracts of canola seed.

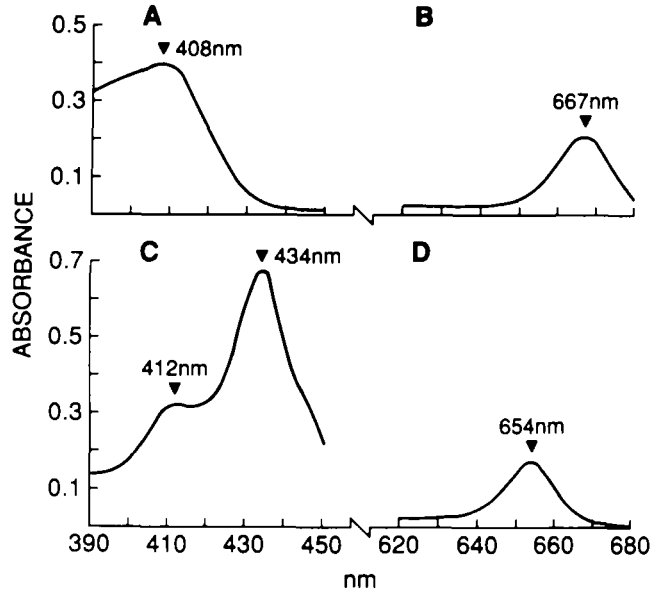

FIG. 8. Absorption spectrum of *pheophytin a* (a,b) and *pheophytin b* (c,d) fractions collected from HPLC analysis of crude canola oil.

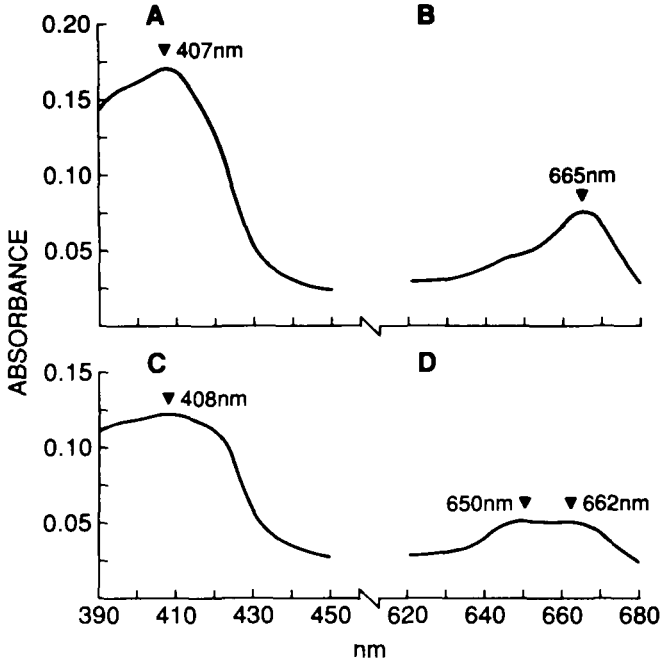

FIG. 9. Absorption spectrum of fractions collected between 4-8 mins (a,b) and between 14-17 mins (c,d) from HPLC analysis of crude canola oil.

ies cited above. Analysis of crude soybean oil (Figure 6 and 7), showed almost no chlorophyll or pheophytin, in agreement with Fraser and Frankel (23).

When different fractions of the chromatogram were collected and analyzed on the scanning spectrophotometers, the identities of the *chlorophyll* and *pheophytin a* and *b* peaks from both the seed and the oil samples were confirmed (Figures 7,8). For certain canola oils, significant absorbances in the 600 to 700 nM region were found in the HPLC fractions eluting between 4-8 min and also between the 14 to 17 minutes (Figure 9). These components need further identification but they could be pheophorbides or chlorophyllides.

Since *pheophytin a* appeared to be the major component present in the crude canola oils studied, a spectrophotometric procedure was developed with *pheophytin a* as a calibration standard. This calibration gave results about 1.5 times higher than the AOCS Method CC 13d-55 as modified by Yuen and Kelly (16) (Figure 10). A similar calibration using *chlorophyll a* gave a 1:1 correspondence with the AOCS method. Total pigments determined by the pheophytin calibration were found to give similar results to the *pheophytin a* or total chlorophylls and pheophytins determined by HPLC (Figure 11).

This study, and the study reported by Fraser and Frankel on soybean oil, show that the current AOCS method for determination of chlorophyll is inaccurate and unreliable. Proposed improvements include calibration for the major pheophytin-based pigments actually present in the oil. It would also be useful to delete references to specific spectrophotometers, most of which are no longer available or found in laboratories, and to write the method in a form applicable to modern spectrophotometers.

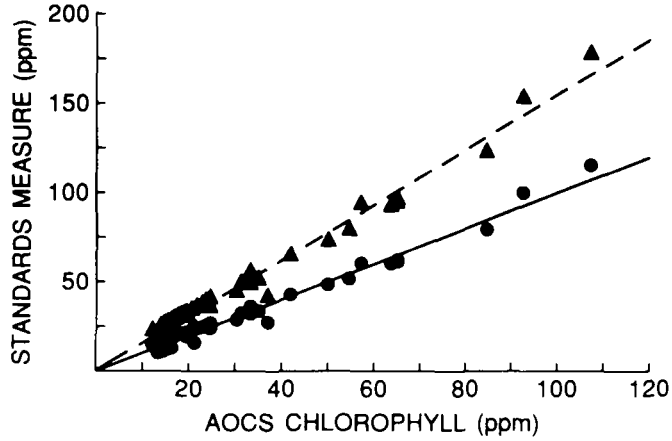

FIG. 10. Spectrophotometric determination of pigments in canola oil. Modified AOCS Method compared to calibrations with *chlorophyll a* (•) and with *pheophytin a* (▲).

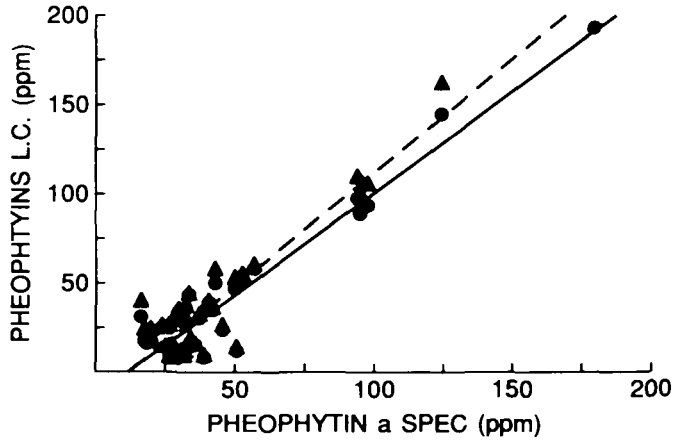

FIG. 11. Comparison of spectrophotometric determination of *pheophytin a* in canola oil to the HPLC determination of *pheophytin a* (•) or total chlorophylls and pheophytins (▲).

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