Characterization of Chlorophyll Pigments in Ripening Canola Seed (*Brassica napus*)

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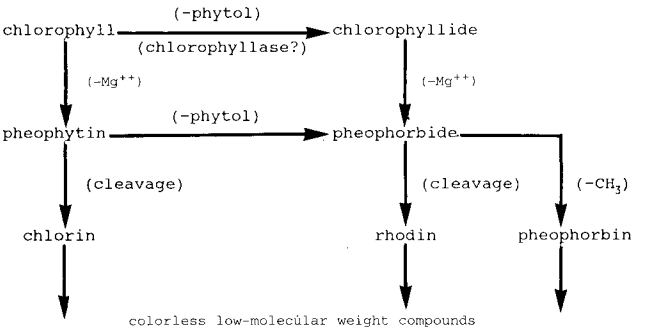
This study characterizes the chlorophyll pigments in ripening *Brassica napus* seed. Seed samples, collected weekly as the crop ripened, were analyzed by high-performance liquid chromatography to characterize chlorophyll pigment composition. Chlorophyll A, chlorophyll B, pheophytin A and pheophytin B were the predominant pigments, while pheophorbide A, methylpheophorbide A and pyropheophytin A were minor components. No differences in pigment composition were observed between the three cultivars tested or between early and late seeding dates. There were differences in pigment composition between the two years of the study, which may result either from seed aging during storage or from environmental influences. Pigment composition was dependent on seed maturity, with physiologically mature green seeds containing both chlorophylls and pheophytins, but fully mature seeds containing only chlorophylls. Pheophytins and the minor components appeared transiently, presumably formed from the chlorophylls and subsequently degraded. The ratio of chlorophyll A/B increased during seed ripening, with fully mature canola seed having a chlorophyll A/B ratio twice that of physiologically mature green seed. The "B" derivatives degraded faster than the "A" derivatives, suggesting enzymatic reactions. The initial steps in the chlorophyll breakdown pathway in canola seed appear to be:

chlorophylls → pheophytins → pyropheophytins
↓
pheophorbides
↓
methylpheophorbides

KEY WORDS: Canola seed, chlorophyll, chlorophyll analysis, chlorophyll breakdown pathway, chlorophyll catabolism, green seed, high-performance liquid chromatography, pheophytin, pigment, ripening.

The leading cause for downgrading of Canadian canola is the presence of unacceptable levels of immature green seed. When the seed is crushed, chlorophyll pigments are extracted with the oil, producing a dark color, which is aesthetically unappealing to consumers. Chlorophyll pigments act as photosensitizers, promoting oxidation of the oil and reducing its shelf life (1–5). These pigments also act as catalyst poisons' which block the active site of the nickel catalyst and impair hydrogenation (6). Chlorophyll can be removed from the oil by adsorption to bleaching clay. Bleaching clay also retains 1/3 to 3/4 of its weight in oil (7), and as chlorophyll levels increase, more bleaching clay is required and oil losses increase.

Chlorophyll breakdown in the seed is poorly understood. The proposed initial steps in the chlorophyll breakdown pathway are illustrated in Scheme 1 (8). The term "chlorophyll" is often used in the general sense to refer to all green pigments in canola seed. Previous studies have indicated that ripe canola seed contained mainly chlorophyll A (CHL A) and chlorophyll B (CHL B) in an approximate 3:1 ratio



SCHEME 1

(9,10), while moldy, heated or otherwise damaged seeds contained fewer chlorophylls and more pheophytins (11).

In recent studies (9,10), high-performance liquid chromatography (HPLC) was used to identify and quantitate the chlorophyll pigments present in commercially harvested canola seed. We used HPLC to characterize the chlorophyll pigments present in canola seed that was harvested at different stages throughout the ripening period. The purposes of this study were to determine which chlorophyll derivatives occur in ripening canola seed and in what proportions, whether the pigment composition changes as the seeds ripen, whether there are differences in pigment composition between seeds of different *Brassica napus* cultivars, and whether the environment affects the composition of chlorophyll derivatives in the seed. With this information, we hoped to elucidate the initial steps in the pathway(s) of chlorophyll breakdown in ripening canola seed.

MATERIALS AND METHODS

Seed samples. Three cultivars of oilseed rape (*B. napus* L.)—Stellar, Westar and Delta—were grown in a randomized complete-block design in two years (1991 and 1992), with four replicates in 1991 and five in 1992, with early and late seeding dates each year. A previous study determined that Stellar seed consistently contained high chlorophyll levels at harvest; Delta produced seed containing little chlorophyll; and Westar was intermediate (12). These three *B. napus* cultivars have distinct genetic backgrounds. Stellar is a low-linolenic canola cultivar developed at the University of Manitoba (Winnipeg, Manitoba, Canada) in 1987 from a mutation line backcrossed to Regent. Delta is a Swedish canola cultivar, developed at the Weibullsholm Plant Breeding Institute (Landskrona, Sweden) in 1989, and Westar is a canola cultivar, developed from a backcross to Tower, released by the Saskatoon Agriculture Canada research station (Saskatoon, Saskatchewan, Canada) in 1982.

Each plant in the inner rows of each plot was given a color-coded tag at first flower. Plants that flowered within a one-week period were sampled to eliminate unusually early or late material. For the early seeding date in 1992,

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a two-week period was allowed because flowering was delayed by frequent rains. Plots were sampled weekly in 1991, beginning at growth stage 5.3 on the Harper and Berkenkamp (HB) scale (13), and at growth stage 5.2 in 1992 (physiologically mature green). Sampling continued until the plants were senescent (HB 5.5). Ten plants were cut for each sample; the main stems were removed and taken to the lab, where the seeds were immediately removed from the pods, weighed, frozen, freeze-dried and stored in sealed glass vials until analysis. In the first year of the study, samples were analyzed after 18 to 21 mon of storage, while the samples harvested in the second year of the study were analyzed within 3 to 8 mon.

Chlorophyll pigment analysis. Two grams of freeze-dried seed were placed in stainless-steel extraction tubes with three ball bearings and 30 mL of 3:1 isooctane/ethanol. Samples were shaken for one hour and filtered, and the extract containing the chlorophyll was collected. High-chlorophyll samples were diluted if necessary to prevent peaks from overlapping, and results were adjusted accordingly. Samples were evaporated to dryness under nitrogen at room temperature and resuspended in acetone.

HPLC analysis was carried out as described in a previous paper (14). A 10–50-μL injection volume was used, depending on the concentration of chlorophyll pigments in the sample. A 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.

RESULTS AND DISCUSSION

The concentration (mg kg^{-1}) of each chlorophyll pigment was determined in each seed sample. The relative proportion (percentage) of each pigment as a percentage of total chlorophyll was averaged over replicates to allow comparison between seed samples with a wide range of chlorophyll levels.

The three cultivars tested—Stellar, Delta and Westar—all contained the same types of chlorophyll pigments—chlorophylls, pheophytins, pheophorbides, methylpheophorbides and pyropheophytins. No pigments were detected that were unique to any one cultivar, and none of the pigments occurred at exceptionally high or low levels in a single cultivar. Because the same breakdown intermediates were detected, chlorophyll likely degrades by the same pathway in all cultivars tested. There were no differences in the types of pigments detected in the early and late seeding dates within the same year (Table 1).

There were differences in pigment composition between the two years of the study. In 1991, the main pigments detected were CHL A, CHL B, pheophytin A (PHY A) and pheophytin B (PHY B). Minor pigments included pheophorbide A (PHO A), methylpheophorbide A (METHYL A) and pyropheophytin A (PYRO A). In 1992, CHL A, CHL B and PHY A appeared as major pigments, but no PHY B was detected. Low levels of PHO A and METHYL A were detected in some samples, but no PYRO A. No unidentified peaks appeared in any samples in either year of the study.

The differences in pigment composition between the two years of the study could be due to seed aging in storage. The freeze-dried samples harvested in 1991 were stored for 18 to 21 mon prior to analysis, while samples collected in 1992 were only stored for 3 to 8 mon. A previous study indicated that frozen canola seed could be stored for up to one month with no change in total chlorophyll, measured by spectrophotometric absorbance (15), but chlorophyll pigment composition was not examined. Prolonged storage of freeze-dried seed might have allowed the seed to deteriorate, and moldy, heated or otherwise damaged seeds have been shown to contain elevated levels of pheophytins (11). PHY A was present at much higher levels in samples from 1991, and PHY B was only detected in samples collected in 1991.

Environmental effects might also explain the differences in pigment composition between the two years of the study. Rates of total chlorophyll breakdown in ripening *B. napus* seed and the influence of environment on chlorophyll breakdown rates have been characterized in a separate paper (Ward, K., R. Scarth, J.K. Daun and J.K. Vessey, unpublished data). In 1991, the seeds ripened quickly during hot, dry weather, with the late seeding date fully ripe by the end of August. In 1992, cool, wet weather delayed ripening and prolonged the sampling period until early October for the late seeding date. From this study, it cannot be determined whether the differences in pigment composition between the two years were caused by seed aging in storage or environmental influences.

TABLE 1

Relative Proportion of Each Chlorophyll Pigment, as a Percentage of Total Chlorophyll, Detected in Physiologically Mature Green Canola Seed (HB 5.3) of Three Cultivars of *Brassica napus* (Westar, Delta and Stellar) Harvested in 1991[a]

	Early seeding date			Late seeding date		
Pigment	Westar (%)	Delta (%)	Stellar (%)	Westar (%)	Delta (%)	Stellar (%)
PHO A	2.2	6.0	2.0	2.8	3.4	4.5
METHYL A	3.2	2.6	0.8	5.5	4.0	17.7
CHL B	22.2	31.3	32.5	22.4	26.6	21.1
CHL A	19.5	24.1	31.8	21.3	24.4	19.4
PHY B	8.5	3.0	2.0	7.7	6.0	8.1
PHY A	43.1	32.0	29.8	38.5	34.4	27.4
PYRO A	1.2	0.9	1.0	1.7	1.2	1.9

[a]Values were averaged over four replicates. PHO = pheophorbide, METHYL = methylpheophorbide, CHL = chlorophyll, PHY = pheophytin and PYRO = pyropheophytin; HB = Harper and Berkenkamp scale (Ref. 13).

The lack of any unidentified peaks at chlorophyll-specific wavelengths in the chromatograms indicates that all chlorophyll breakdown products that can be detected by the chosen HPLC system were either PHO A, METHYL A, CHL B, CHL A, PHY B, PHY A or PYRO A. This suggests that the initial steps in the chlorophyll breakdown pathway in ripening canola seed may be:

$$\begin{array}{l} \text{CHL A} \rightarrow \text{PHY A} \rightarrow \text{PYRO A} \rightarrow ? \\ \qquad\quad \downarrow \\ \qquad\quad \text{PHO A} \rightarrow \text{METHYL A} \rightarrow ? \\ \qquad\quad \text{and CHL B} \rightarrow \text{PHY B} \rightarrow ? \end{array}$$

A series of investigations by Johnson-Flanagan and colleagues (16–18) have attempted to determine the pathway(s) and enzymes involved in chlorophyll breakdown in ripening canola seeds by means of cultured embryos. CHL A and CHL B were the main pigments detected, with low levels of pheophytins, pheophorbides and chlorophyllides. During ripening, both chlorophyllide A and PHY A accumulated, indicating that two separate pathways operate simultaneously in degreening canola seed (16–18). We were unable to detect chlorophyllide or evidence of a second catabolic pathway in our study.

The mean level of each chlorophyll pigment was determined over replicates, cultivars and seeding dates in each year of the study (Tables 2 and 3). Table 4 shows the changes in chlorophyll pigment composition that occur as canola seed ripens from physiologically mature green (HB 5.2) to full maturity (HB 5.5). This pattern was similar for all three cultivars and both seeding dates of each year. The earliest sample collected from each plot (physiologically mature green seed) averaged 1166 mg kg^{-1} total chlorophyll in 1991 and 962 mg kg^{-1} in 1992. The fully mature seed from the final sample taken from each plot averaged 2 mg kg^{-1} total chlorophyll in 1991 and 1 mg kg^{-1} in 1992. It is clear that pigment composition depends on seed maturity. Physiologically mature green seeds (HB 5.2–5.3) contained significant amounts of CHL A, CHL B, PHY A and PHY B, but at maturity (HB 5.5), canola seeds contained mainly CHL A and CHL B. The minor components (PHO A, METHYL A and PYRO A) occurred sporadically as the seed matured, but most had disappeared when the seed reached full maturity (Table 4). These minor components were likely transient breakdown products that appeared as CHL A degraded. PHY A and PHY B appeared only in physiologically mature green seed and disappeared within three to four weeks as the seeds ripened (Table 4). Pheophytins appear to be temporary breakdown products, formed from CHL A or B, which are then further degraded. The transient nature of the pheophytins is confirmed by following the ratios of pheophytin/chlorophyll during seed ripening (Table 4). In all cultivars, seeding dates and years tested,

TABLE 2

Relative Proportion of Each Chlorophyll Pigment, as a Percentage of Total Chlorophyll, Detected in Physiologically Mature Green (HB 5.3) and in Fully Mature (HB 5.5) Canola Seed Harvested in 1991[a]

	Green seed		Fully mature seed	
Pigment	Average (%)	Range (%) (averaged over replicates)	Average (%)	Range (%) (averaged over replicates)
PHO A	3.5	2.0–6.0	0.2	0.0–1.0
METHYL A	5.6	0.8–17.7	0.5	0.0–1.0
CHL B	26.0	21.1–32.5	32.1	17.1–56.0
CHL A	23.3	19.4–31.8	66.0	44.0–82.4
PHY B	5.8	2.0–8.5	—	0.0–trace
PHY A	34.2	27.4–43.1	1.3	0.0–3.9
PYRO A	1.3	0.9–1.9	—	0.0–0.0

[a]Average values were calculated over four replicates, three cultivars (Stellar, Delta and Westar) and both early and late seeding dates. See Table 1 for abbreviations.

TABLE 3

Relative Proportion of Each Chlorophyll Pigment, as a Percentage of Total Chlorophyll, Detected in Physiologically Mature Green (HB 5.2) and Fully Mature (HB 5.5) Canola Seed Harvested in 1992[a]

	Green seed		Fully mature seed	
Pigment	Average (%)	Range (%) (averaged over replicates)	Average (%)	Range (%) (averaged over replicates)
PHO A	0.2	0.0–0.4	0.3	0.0–1.8
METHYL A	trace	0.0–0.1	1.0	0.0–3.5
CHL B	28.4	26.2–30.3	19.9	15.1–24.4
CHL A	63.8	61.3–65.4	78.7	72.1–84.9
PHY A	6.8	5.1–10.5	0.1	0.0–0.8

[a]Average values were calculated over five replicates, three cultivars (Stellar, Delta and Westar) and both early and late seeding dates. See Table 1 for abbreviations.

TABLE 4

Relative Proportion of Each Chlorophyll Pigment, as a Percentage of Total Chlorophyll, Detected in Ripening Canola Seed (cv. Westar) from the Early Seeding Date of 1991[a]

	Days from start of sampling					
Pigment (%)	1	6	14	20	27	35
PHO A	2.2	1.7	0.6	1.5	—	—
METHYL A	3.2	3.5	1.8	0.5	3.0	0.5
CHL B	22.2	22.1	15.8	27.3	54.1	17.1
CHL A	19.5	23.4	27.2	58.7	41.9	82.4
PHY B	8.5	7.4	11.3	2.0	—	—
PHY A	43.1	39.8	40.9	10.0	1.1	—
PYRO A	1.2	2.3	2.5	trace	—	—
Total chlorophyll (mg kg^{-1})	1239	906	463	48	8	4
% "A" derivative	69.3	70.6	72.9	70.7	46.0	82.9
CHL A/B	0.88	1.06	1.72	2.15	0.77	4.82
PHY A/B	5.07	5.38	2.62	5.00	PHY A	—
PHY B/CHL B	0.38	0.33	0.72	0.073	CHL	CHL
PHY A/CHL A	2.21	1.70	1.50	0.17	0.026	CHL

[a]PHY A indicates that no PHY B was detected, CHL indicates that only chlorophylls were detected. See Table 1 for abbreviations.

the ratio of PHY B/CHL B and of PHY A/CHL A declined as the seed matured from physiologically mature green (HB 5.2) to full maturity (HB 5.5). In the final seed samples taken from each plot, only chlorophylls remained.

Endo *et al.* (10) examined commercial canola seed from a crushing plant and found CHL A and CHL B to be the main pigments present, with PHY A present as a minor component and traces of PHO A and METHYL A in some samples. We also found CHL A and CHL B to be the major pigments in fully mature canola seed, but ripening seed also contained PHY A and PHY B and traces of PHO A, METHYL A and PYRO A. Ours is the first report of PHY B and PYRO A in canola seed. As these pigments were only observed in one year of the study, it is uncertain whether these compounds occurred naturally in ripening seeds, or whether they were formed during seed storage.

The percentage of "A" derivatives detected varied with the stage of seed maturity from 44 to 87%. In general, the percentage of "A" derivatives in fully mature seed was higher than in physiologically mature green seed (Table 4), but the transient appearance of the pheophytins caused much variability. Results were more consistent when the ratio of CHL A/B was followed during seed ripening. In 1991, the CHL A/B ratio in physiologically mature green seed was approximately 1:1, while in fully mature seed it was close to 2:1. In 1992, physiologically mature green seed averaged a CHL A/B ratio of approximately 2:1, while in fully mature seed this value was close to 4:1. Therefore, the CHL A/B ratio approximately doubled as canola seed ripened from physiologically mature green (HB 5.2) to full maturity (HB 5.5). In previous studies by Daun and Thorsteinson (9) and by Endo *et al.* (10), the CHL A/B ratio in commercial samples of canola seed was 3:1. Ours is the first study to show that the ratio of CHL A/B depends on the stage of maturity of the seed.

CHL B degraded faster than CHL A, suggesting an enzymatic reaction because nonenzymatic photobleaching degrades CHL B and CHL A at the same rate (19). Researchers working with other plant species have found that, during plant senescence, the ratio of CHL A/B decreases, with CHL A being degraded more quickly (20). However, in ripening *B. napus* seed, this was not the case. CHL B degraded faster than CHL A, with an accompanying rise in the CHL A/B ratio as the seed matured (Table 4). The PHY A/B ratio showed a similar pattern to the chlorophylls in 1991, where this could be determined, with PHY B disappearing sooner than PHY A.

The amount and composition of chlorophyll pigments in the seed determines what will end up in the oil. Oil extraction and processing alters these pigments, with chlorophylls being converted to pheophytins and pyropheophytins (10,14). The chlorophyll pigment composition of the oil affects the bleaching process and oil stability. There is little published information on the relative ease of removal of each pigment during bleaching. Knowledge of the chlorophyll pigment composition of canola seed and how these pigments are altered during oil processing must be combined with bleaching studies to determine the best way to remove each pigment from the oil. This should eventually allow crushers to manipulate processing conditions to yield an oil that can be bleached efficiently to yield a stable saleable oil.

ACKNOWLEDGMENTS

Financial support was provided by the Canadian Wheat Board, the University of Manitoba Graduate Fellowship and the J.S. Lightcap Award. Materials, equipment and technical assistance were generously supplied by the staff at the Grain Research Lab of the Canadian Grain Commission. This paper is contribution number 968 and 724.

REFERENCES

1. Usuki, R., Y. Endo, T. Suzuki and T. Kaneda, *Proceedings of 16th ISF Congress*, Budapest, 1983, p. 627.
2. Endo, Y., R. Usuki and T. Kaneda, *J. Am. Oil Chem. Soc. 61*:781 (1984).
3. Usuki, R., Y. Endo and T. Kaneda, *Agric. Biol. Chem. 48*:991 (1984).
4. Endo, Y., R. Usuki and T. Kaneda. *Ibid. 48*:985 (1984).
5. Kiritsakis, A., and L.R. Dugan, *J. Am. Oil Chem. Soc. 62*:892 (1985).
6. Abraham, V., and J.M. deMan, *Ibid. 63*:1185 (1986).
7. Mag, T.K., *World Conference Proceedings—Edible Fats and Oils Processing: Basic Principles and Modern Practices*, American Oil Chemists' Society, Champaign, 1990, p. 107.
8. Humphrey, A.M., *Food Chemistry 5*:57 (1980).
9. Daun, J.K., and C.T. Thorsteinson, *J. Am. Oil Chem. Soc. 66*:1124 (1989).
10. Endo, Y., C.T. Thorsteinson and J.K. Daun, *Ibid. 69*:564-568 (1992).
11. Johansson, S.-A., and L.-A. Appelqvist, *Fette Seifen Anstrichm. 8*:304 (1984).
12. Ward, K.A., *The Effect of Genotype, Environment and Agronomic Practices on the Chlorophyll Level in Harvested Canola Seed*, Thesis, University of Manitoba, Winnipeg Manitoba, 1990.
13. Harper, F.R., and B. Berkenkamp, *Can. J. Plant Sci. 55*:657 (1975).

14. Ward, K., R. Scarth, J.K. Daun and C.T. Thorsteinson, *J. Am. Oil Chem. Soc. 71*:931 (1994).
15. Ward, K., R. Scarth and J.K. Daun, *Ibid. 69*:1039 (1992).
16. Johnson-Flanagan, A.M., and M.R. Thiagarajah, *J. Plant Physiol. 136*:180 (1990).
17. Johnson-Flanagan, A.M., and G. McLachlan, *Physiol. Plant 80*:453 (1990).
18. Johnson-Flanagan, A.M., and G. McLachlan, *Ibid. 80*:460 (1990).
19. Jen, J.J., and G. MacKinney, *Photochem, Photobiol. 11*:303 (1970).
20. Simpson, K.L., T.-C. Lee, D.B. Rodriguez and C.O. Chichester, *The Chemistry and Biochemistry of Plant Pigments*, 2nd edn., Vol 1, edited by T.W. Goodwin, Academic Press, New York, 1976, pp. 780–797.

[Received March 28, 1994; accepted September 10, 1994]