Chemical Aspects of Chlorophyll Breakdown Products and Their Relevance to Canola Oil Stability

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The production of prooxidant compounds brought about through subjecting chlorophyll a or pheophytin a to laboratory-scale processing in the presence of canola oil or tricapryloylglycerol was investigated. The addition of chlorophyll a (60 ppm) to canola oil prior to processing resulted in an oil of lowered stability. No large contribution to the produced instability by any one processing step was found when pheophytin a was added (60 ppm) to canola oil prior to processing. To isolate the effect of processing on the pigment, tricapryloylglycerol was used in the place of unsaturated canola oil as a carrier for pheophytin a (60 ppm). A control consisted of processed tricapryloylglycerol that had no added pheophytin prior to processing. The subsequent addition of pigment-treated processed tricapryloylglycerol to linseed oil (1:1, w/w) caused a decrease in the stability of the latter, when compared with the control. No differences were observed between the prooxidant tricapryloylglycerol and the control tricapryloylglycerol by methods involving ultraviolet spectroscopy and thin-layer or gas chromatography.

KEY WORDS: Autoxidation, canola oil, chlorophyll, pheophytin, processing, stability.

Chlorophyll is a ubiquitous plant pigment. In vegetable oil processing this pigment is converted to a structurally similar green pigment known as pheophytin (1). The levels of pheophytin in canola oil are dependent upon several factors, including the maturity of the crop at the time of harvest and the extent of any frost damage. Pheophytin levels are normally in the range of 15–30 parts per million in crude canola oil. This level is significantly higher than that found in other vegetable oils and is the largest single quality impediment for Canadian canola oil. The high levels of pheophytin represent a marketing deterrent as they must be removed to the parts per billion range in finished oil products. To do this the oil processor must use high levels of clay absorbent, which increases processing costs, thereby putting Canadian canola oil at an economic disadvantage.

Although most Canadian oil refineries have the technology to remove pheophytin to the parts per billion range, there are questions on the oxidative and light stability of oils derived from high-chlorophyll seed. Coe (2) first reported the correlation between chlorophyll content and the extent of oxidative deterioration of oils. Since this first investigation a number of researchers have shown that chlorophyll and its breakdown products, pheophytin and pheophorbide, can act as prooxidants in oils (3–5). While there has been an established association between oxidative stability and chlorophyll and pheophytin levels, the question of the cause for reduced stability after pigment removal in refined oils remains unanswered.

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Jackson (6) postulated that exposure of chlorophyll to chemicals, oxygen or enzymes would result in the formation of colorless low-molecular weight compounds. Except for the breakdown products pheophytin, pheophorbide, chlorophyll and chlorin (each of which is a macromolecule with an absorbance >400 nm), this postulate has not been investigated.

The breakdown of chlorophyll a and b in senescing tissue has been the subject of a number of recent publications (7,8). In each of these studies pheophytin a and b and pheophorbide were isolated; however, further breakdown products were not isolated. Published ultraviolet (UV)-visible spectra in one of these papers (7) indicated the presence of a compound(s) that absorbed in the 260–350 nm region.

The purpose of this study was to determine the cause behind the decreased stability of greenseed oils. It was proposed that a likely factor is the presence of prooxidant breakdown products of pheophytin that are produced through processing and remain in the final oil. Investigations were undertaken to determine the identity of any such breakdown products, thereby facilitating the production of means to decrease or eliminate such compounds in the final product.

MATERIALS AND METHODS

Tricapryloylglycerol (95% pure) and chlorophyll a (99% pure) were obtained from Sigma Chemical Co. (St Louis, MO). Fully processed canola oil was purchased commercially and further purified *via* silica column chromatography.

Preparation and addition of pigments to oil. All treated oils used in these experiments were combined with either chlorophyll a or pheophytin a at a level of 60 ppm. In experiments involving chlorophyll a, 1 mg of this compound was solubilized in 1 mL of acetone prior to addition to 15 g of oil. Any oils labelled as a control were mixed with 1 mL of acetone with no added chlorophyll a. In experiments involving pheophytin a, the preparation of this compound involved dissolving 1 mg of chlorophyll a in 1 mL of acetone, followed by the dropwise addition of 13% HCl until an obvious color change was apparent. Hexane (1 mL) was added, followed by the addition of 1 mL of deionized water. The nonpolar layer containing the pheophytin was removed, washed with 1 mL of deionized water (5 × 1 mL) and then added to a known weight of oil. The pheophytin a control consisted of the same steps of acid addition, hexane extraction and water wash, applied to 1 mL of acetone with no added chlorophyll.

Processing conditions. All processing steps were carried out in controlled-temperature oil baths on IKAMAG RET-G stirrer/heaters (Rose Scientific Ltd., Edmonton, Alberta, Canada), with agitation provided through stir bars set at a rapid rotation rate (700 on the stir bar control).

Degumming was carried out at 40°C and involved a 15-min exposure of the oil to 0.2% (vol/wt) citric acid (50%

wt/vol in water), followed by the addition of 2% (vol/wt) deionized water. After a further period of 15 min, the mixture was centrifuged (Sorvall Superspeed centrifuge, SS-1; Newton, CT) at 4400 × *g* for 5 min at 4°C, followed by the removal of the oil layer by pipetting.

Refining involved an initial 1-h pretreatment at 40°C with 0.015% (vol/wt) of orthophosphoric acid (Analar grade, BDH Inc., Edmonton, Alberta, Canada). Sodium hydroxide (Assured grade, BDH Inc.) (9.5% wt/vol) was then added in sufficient volume to neutralize 1.1% free fatty acid. This amount was selected as representative of the high end of typical values encountered in the processing industry (9), and was not based on the content of free fatty acids in the experimental oil, as the latter was a purified product and, as such, was not expected to contain any free fatty acids. After a 15-min exposure at 40°C, the mixture was centrifuged at 4400 × *g* for 5 min, and the oil layer was removed by pipette. The mixture was then washed two times with an equivalent volume of deionized water at 70°C.

Bleaching was accomplished with the use of 5% (w/w) Filtrol 105 clay (Engelhard Corp., Jackson, MS). The mixture was stirred for 30 min at 104°C under a continuous stream of nitrogen. Centrifugation at 4400 × *g* for 15 min was used to remove the clay, as well as a final filtering of the oil through a 0.2-μm Corning filter (BDH, Inc.). The absorbance between 600–700 nm of all oils with pigment added prior to processing was determined (neat) to ascertain the extent of bleaching. If necessary, both the control oil and the treated oil(s) were re-bleached until no absorbance was seen in this region (indicating less than 65 ppb pheophytin a present).

Deodorization was performed in equipment modelled after that described by Moulton (10), with a temperature of 260°C for 1 h (after allowing a 45-min come-up period) at 0.3 mm Hg pressure. All oils were immediately stored at −20°C following deodorization until used.

Absorbance measurements. All measurements were made with a Milton Roy (Rochester, NY) Spectronic model 1201 spectrophotometer on aliquots of the oils solubilized in hexane.

Thin-layer chromatography. Aluminum-backed silica gel plates (E.M. Separations, Gibbstown, NJ) were activated for 1 h at 110°C. Solvent systems were hexane/diethyl ether (70:30, 50:50, 30:70, vol/vol). Visualization was attempted by charring with concentrated sulfuric acid, by the application of 2,7-dichlorofluorescein, followed by viewing under UV light and by exposure of the plates to iodine vapor.

Gas-liquid chromatography. A Varian Model 3400 gas chromatograph was used with a capillary column (HT-5, 0.25 μm film, 3 m × 0.22 mm i.d.; Scientific Glass Engineering, Inc., Austin, TX). The column flow rate was set at 2 mL/min with helium as a carrier gas. Injector and flame-ionization detector (FID) temperatures were 350 and 380°C, respectively. Temperature programming was used with an initial oven temperature of 80°C, followed by a linear increase at a rate of 10°C/min to a maximum temperature of 360°C, which was maintained for 10 min. Sample size was a 1-μL injection of a 2-mg/mL solution of oil in chloroform.

Stability assessment. Aliquots of oil (0.1 g) were distributed in a series of 13 × 100-mm test tubes and placed in a forced-air oven at 60°C. At appropriate time intervals, two test tubes from each treatment were removed, and the absorbance at 234 nm was determined as indicative of conjugated diene content. The absorbances obtained were averaged for the duplicates of each treatment.

When tricapryloylglycerol was used as the carrier for the pheophytin during processing, the effect of any breakdown products was determined by thoroughly mixing equivalent weights of the tricapryloylglycerol and a purified canola oil, followed by the described method for stability assessment.

The effect of the addition of chlorophyll a to canola oil prior to processing. Chlorophyll a was added to purified canola oil to achieve a final level of 60 ppm. The processing steps of degumming, phosphoric acid treatment, refining and bleaching were then applied to both the chlorophyll-treated oil and a control oil, followed by a stability assessment *via* accelerated storage.

The effect of processing step. To determine which, if any, particular processing step was the cause of the decreased stability of pigment-treated oil, canola oil with added pheophytin (60 ppm) was subjected to various treatments that involved exposing the oil to one or more levels of processing: i) bleaching only; ii) refining and bleaching; iii) treatment with phosphoric acid, followed by refining and bleaching and iv) degumming, phosphoric acid treatment, refining and bleaching. A control treatment consisted of canola oil with no added pheophytin subjected to all four processing steps. A UV scan of the produced oils was performed, as well as an assessment of comparative stability.

Effect of addition of pheophytin to tricapryloylglycerol prior to processing. Results of the above two experiments suggested that the unsaturated nature of canola oil may have been the cause of reactions during processing that were independent of any alterations to pheophytin that were occurring at the same time. Because products of both of these reactions may have an effect on the resulting oil stability, it was decided to use a fully saturated oil when investigating the effect of processing on pheophytin. In this manner, it was hoped that the effect of processing on pheophytin could more readily be isolated and ascertained. Tricapryloylglycerol, a fully saturated oil that should be unaffected by any processing step, was then mixed with pheophytin at a level of 60 ppm and subjected to full processing, including deodorization. To further decrease the effect of any reactions due to the photosensitive nature of the pigments and to more nearly duplicate industrial conditions, all processing up to and including bleaching were performed in the dark. The stability assessment was performed on an equivalent-weight mixture of the tricapryloylglycerol with purified linseed oil. The latter was chosen in lieu of canola oil because the period required for stability assessment was significantly shorter.

RESULTS AND DISCUSSION

Effect of chlorophyll a addition to canola oil prior to processing on the stability of the finished oil. Figure 1 presents the results of the stability test performed on canola oil to which chlorophyll a had been added prior to processing, and on a control oil that had no chlorophyll a added prior to processing. The curves shown indicate that the chlorophyll-treated oil exhibited a more rapid increase in the buildup of primary autoxidation products and a shorter induction period. Because the only difference

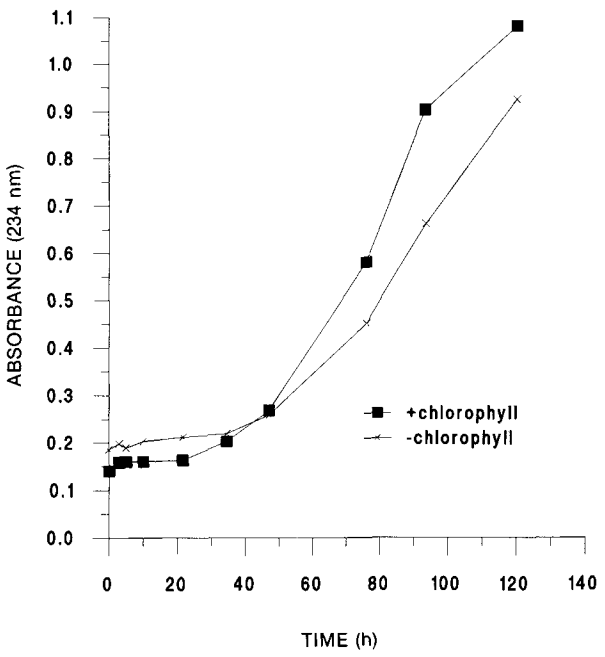

FIG. 1. Absorbance at 234 nm (indicative of conjugated-diene content) as a function of storage at 60°C for canola oil ± chlorophyll a (60 ppm).

between the two oils was the addition of chlorophyll a to the treated oil prior to processing, this result strongly suggests that a prooxidative effect was produced through the interaction of chlorophyll a and one or more of the laboratory processing techniques used. Identification of the particular step that had the major influence in the development of this instability might give important information in revealing how such instability is produced.

Effect of various processing steps on canola oil stability. Since pheophytin had been shown to be the major chlorophyll derivative in crude oil (1), this compound was used instead of chlorophyll in subsequent experiments. Results of the stability tests performed on the oils subjected to various processing procedures are presented in Figure 2. A general trend appeared in the stabilities; oils that had been exposed to more of the processing steps exhibited reduced stability. Similar results were observed by Kwon *et al.* (11) and by Gordon and Rahman (12). However, their reported experiments were performed with crude vegetable oils, and the changes in stability were concluded to be due to the removal or reduction of the contents of various pro- and antioxidants (*e.g.*, phospholipids, free fatty acids, tocopherols). A different case is represented by our experiment because the original oil was purified and thus did not contain the high content of nontriacylglycerol components found in crude vegetable oils.

Figure 2 shows that no single treatment produced substantial reductions in oil stability. The inability to attribute the production of this instability to any particular processing step suggests either gradual breakdown of pheophytin to compounds that would cause instability (not subsequently removed by bleaching), or to the occurrence of autoxidation during the processing period. The latter may have resulted in the production of secondary autoxidation products that can be less stable than triacylglycerols (13) and may not be removed by bleaching. As processing was being conducted with an unsaturated oil combined with a known photosensitizer in the presence

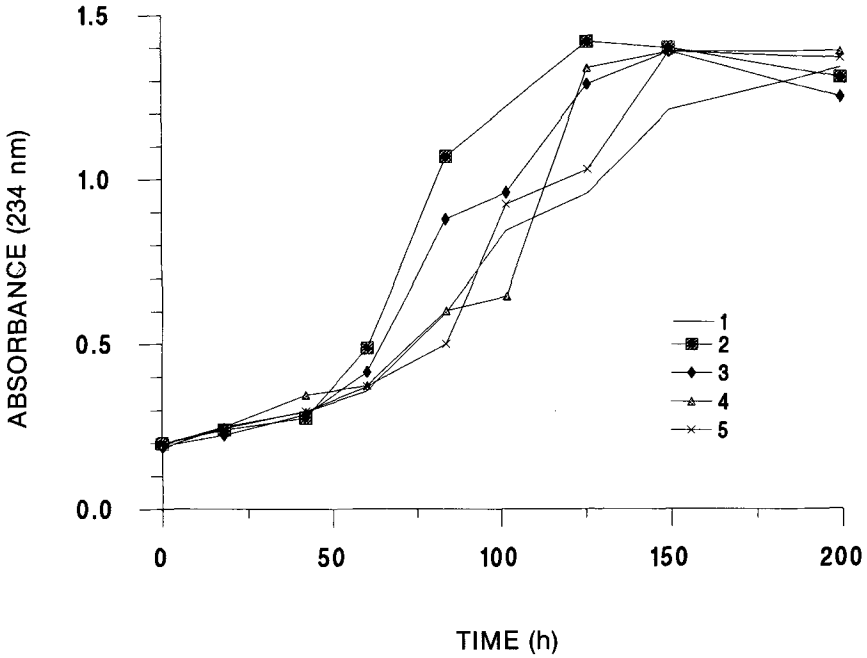

FIG. 2. Absorbance at 234 nm (indicative of conjugated-diene content) as a function of storage at 60°C for canola oil (± pheophytin a, 60 ppm) subjected to various processing steps: 1) all steps (no added pheophytin); all remaining with added pheophytin: 2) all steps; 3) phosphoric acid treatment, refining and bleaching; 4) refining and bleaching; 5) bleaching.

TABLE 1

Absorbance Units of Peak Maxima for Oils Subjected to Various Processing Conditions (values in parentheses are oil concentrations in hexane)

Oil	Processing steps applied	Absorbance units 268 nm (2 mg/mL)	280 nm (2 mg/mL)	315 nm (20 mg/mL)
Control	Degumming, Acid treatment, Refining, Bleaching	0.197	0.156	0.090
Pheophytin-treated	Bleaching	0.190	0.150	0.100
	Refining, Bleaching	0.215	0.170	0.145
	Acid treatment, Refining, Bleaching	0.240	0.195	0.320
	Degumming, Acid treatment, Refining, Bleaching	0.260	0.215	0.390

of light, this was a distinct possibility. To determine whether this was occurring, a UV scan of each of the processed oils was performed from 200 to 400 nm. Table 1 reveals the data obtained from such a scan and shows that as the stabilities of the oils decreased, the absorbance at peak maxima of 268, 280 and 315 nm increased. An increase at these three wavelengths, which are the absorption maxima of conjugated trienes and tetraenes (14,15), suggests the occurrence of autoxidation during the processing period. It was apparent that it would be necessary to ensure that no autoxidation was occurring during the processing period, and this was most readily effected by using a fully saturated triacylglycerol as the carrier for the pigment during processing.

Effect of added pheophytin to tricapryloylglycerol prior to processing. The result of added pheophytin to tricapryloylglycerol prior to processing on the stability of linseed oil is presented in Figure 3. The stability study showed a decrease in the stability of the linseed oil to which the pigment-treated tricapryloylglycerol had been added. Because the only difference between the two oils was the presence of pheophytin a in the treated oil prior to processing, any differences in the effect on linseed stability can be attributed to the presence of the pigment. Use of the saturated triacylglycerol as a carrier for the pheophytin during processing negated any effect of autoxidation occurring during processing, and any prooxidant compounds present in the finished oil would have resulted from the pheophytin itself. It is apparent then that some compound or compounds were produced when pheophytin was subjected to the conditions present during processing that were not removed with further processing steps and that acted as prooxidants. The next step in this study was to determine the identity of such compounds. Several techniques were utilized to determine whether there were detectable compounds present in the pheophytin-treated oil that were not present in the control oil.

The results of absorbance measurements of the pheophytin-treated oil and the control oil in the range of

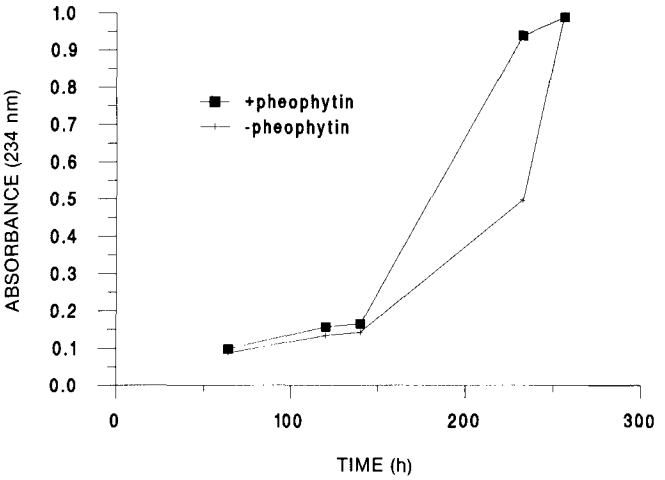

FIG. 3. Absorbance at 234 nm (indicative of conjugated-diene content) as a function of storage at 60°C for tricapryloylglycerol/linseed oil mixtures (1:1, w/w) ± pheophytin a (60 ppm).

200–400 nm is shown in Figure 4. A single peak was observed at 200 nm, which corresponds to the maximum wavelength of absorption for tricapryloylglycerol. No differences in the spectra were observed between the two oils. It is possible that the instability-causing compound(s) either absorb in this region and are obscured by the large absorbance of the tricapryloylglycerol, or do not absorb in the UV region. In addition, it is also possible that the method employed was not sensitive enough to detect these compounds.

Thin-layer chromatography was used in an attempt to separate any instability-causing compounds from the oils. Several mobile phases were tried that varied in their polarity, and several visualization methods were utilized. However, no combination of solvent system and visualization technique revealed any compounds separated from

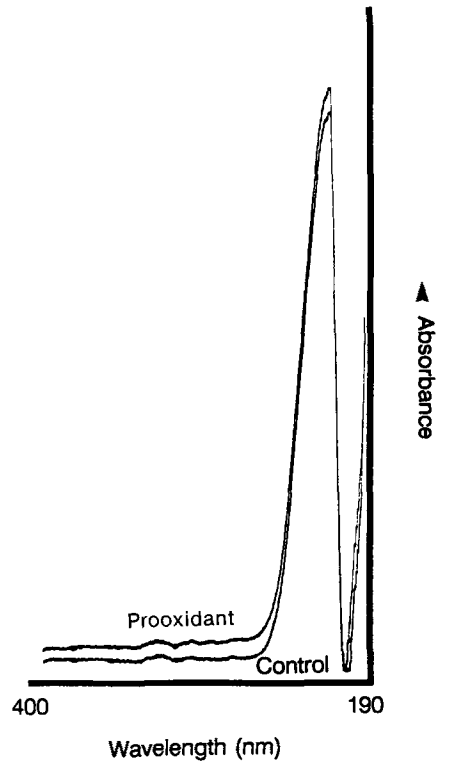

FIG. 4. Ultraviolet spectra of prooxidant and control tricapryloylglycerol.

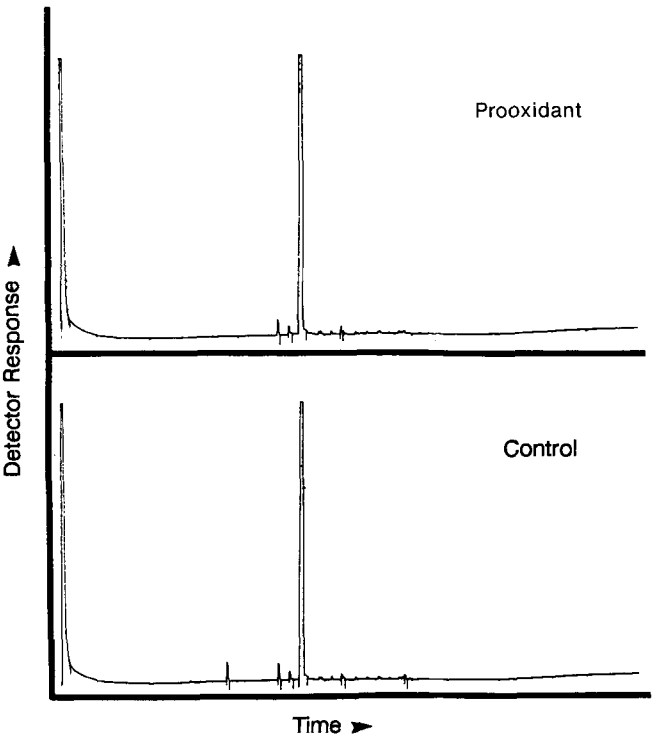

FIG. 5. Gas chromatograms of prooxidant and control tricapryloylglycerol.

the one with an R_f identical to tricapryloylglycerol. As in the previous method, it was possible that either the compounds were present in the pheophytin-treated oil but were not separated by the methods used or the visualization techniques were not appropriate to the compounds or were not sensitive enough for detection.

Finally, gas-liquid chromatography was tried to detect prooxidant breakdown products in the pheophytin-treated tricapryloylglycerol. Figure 5 presents typical chromatograms obtained for pheophytin-treated oil and the control. One major peak appeared in both chromatograms, corresponding to the retention time of tricapryloylglycerol. No unique peaks were present in the pheophytin-treated oil that were not present in the control oil. Again, this indicates that the method either did not effect the separation of the breakdown products, or that the FID was incapable of detecting them at the levels injected.

The results of the stability testing of oils treated with chlorophyll derivatives prior to processing showed that the initial presence of these compounds resulted in a processed oil of lower stability. Such a result is most likely due to the conversion of the chlorophyll derivative to compounds that are capable of being prooxidants. This may not be the only cause of the lower stability of oils processed from seed with a high chlorophyll content but it is certainly a contributing factor. It is apparent that there is a need for further research in this area to isolate and identify such compounds with the final aim of devising some means to prevent their formation or to remove them from the finished oil.

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