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Chlorophyll Degradation and Formation of Colorless Chlorophyll Derivatives during Soybean (*Glycine max* L. Merill) Seed Maturation

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The natural chlorophyll degradation results in noncolored chlorophyll catabolites (NCCs), but there are controversies if these are the final products. The formation and degradation of NCCs during soybean seed (*Glycine max* L. Merrill) maturation and two drying temperatures were investigated. Soybean was harvested at six maturation stages. The effect of postharvest drying at 40 and 60 °C on the NCC formation was analyzed by high-performance liquid chromatography (HPLC), and results were expressed as areas under the curve. All samples contained fractions with an absorption maximum at 320 nm, typical for NCC. The amounts of NCC increased until 114 days after planting and were significantly lower in advanced maturation stages. These results indicate that the NCC in soybeans might not be the final products of chlorophyll degradation. Their reduction in advanced maturation stages may be due to further metabolization. Heating soybeans at 40 and 60 °C promoted unnatural chlorophyll degradation and impaired the formation of NCC.

KEYWORDS: Soybean; *Glycine max* L. Merill; chlorophyll degradation; noncolored chlorophyll catabolites; postharvest drying

INTRODUCTION

In soybean, the chlorophylls are completely degraded to colorless derivatives under normal maturing and drying conditions (*1*). The initial degradation process results in brown-green catabolites, which still possess photodynamic properties because of the presence of an intact tetrapyrrole macrocycle. The natural breakdown starts with the hydrolysis of the phytol group of either chlorophyll *a* or chlorophyll *b* by chlorophyllase, forming chlorophyllides and removal of the magnesium atom by Mg-dechelatase, forming pheophorbides. The final degradation steps involve the formation of colorless products, fluorescent or nonfluorescent, which differ from each other in polarity and the localization of the double bonds in the tetrapyrrole ring (*2*). Green plants are able to adapt their photosynthetic functions according to the light intensity exposure, transforming chlorophyll *b* into chlorophyll *a* with the help of the enzyme chlorophyll *b* reductase (*3, 4*). This transformation is also crucial for the degreening of chlorophyll *b*, because pheophorbide *a* monooxygenase (PaO), which leads to the oxygenolytic opening of the porphyrinoid macrocycle, is specific for pheophorbide *a* (*1, 5*). The PaO produces the red chlorophyll catabolites (RCCs), which are further modified by the RCC reductase that hydrogenates a double bond in the tetrapyrrole ring forming colorless, fluorescent catabolites (FCCs) (*6*). Finally, deconjugation of double bonds of the tetrapyrrolic ring in an acidic medium leads to the formation of nonfluorescent chlorophyll catabolites (NCCs) without participation of any known enzyme (*7*). This catabolism can be understood as a detoxifying process for the plant because of the loss of photodynamic and prooxidant properties of chlorophylls (*8, 9*), and the NCCs are considered the final products of chlorophyll degradation, stored in the cell vacuoles (*7, 10*). However, this accumulation is still controversial given that other tetra- and monopyrrolic structures were found, indicative of further degradation of NCCs. It has been suggested that the NCCs could be precursors of substances crucial for seed germination (*4*). For example, colorless urobilinogen-like linear tetrapyrrols were identified in degreened primary leaves and cotyledons of barley (*11, 12*). A recent study also showed that NCCs are present in the peel of pears and feature effective antioxidant properties. They impaired the formation of linoleic acid hydroperoxides induced by azoisobutyronitrile, because of their peroxy radical scavenging effect. While the fate of the NCCs in fruits is still unknown, authors suggest that they may exert physiological functions in senescent plants and fruits (*13*).

Soybean, submitted to postharvest heating and drying processes mostly by applying hot and dry air or steam to reduce moisture contents for safe storage, may have their natural chlorophyll degradation partially blocked. Drought stress in the field, premature harvesting followed by fast drying processes at high temperatures, or application of desiccants to anticipate

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harvesting are factors that may stop the progress of degreening (*14*). These unfavorable external climatic factors, agricultural practices, and postharvest treatments may affect the pathway, rate, and amounts of residual chlorophyll. In these cases, the major degradation pathways of chlorophylls seem to occur through complex chemical reactions, given that enzymes are totally or partially inactive. Pheophytinization seems to predominate and may be the result of disorganization of cell membranes in acidic pH; the transformation of chlorophylls and the observed accumulation of pheophytins are consistent with this hypothesis. The presence of dephytilated chlorophyll derivatives, such as chlorophyllides and pheophorbides, is not expected because enzymes are mostly inactive and also further degradation to colorless derivatives might be impaired.

The objective of the present work was to study for the first time the behavior of chlorophyll degradation in soybeans with an emphasis on the fate of the NCCs. To find out if these last chlorophyll degradation products are stored or further degraded, we adopted a study design involving the analysis of soybean samples harvested at different stages of development, analyzed freshly and after drying at two temperatures, where chlorophyll degradation was prejudiced. The effect of these treatments on the NCC formation and degradation was reported.

MATERIALS AND METHODS

Soybean Samples. The soybean (*Glycine max* L. Merrill) cultivar IAC-18 was developed and produced under controlled conditions at the Agronomic Institute of Campinas (IAC), similar to those mentioned in a previous study (*14*). Seeds in their intact pods were harvested during the reproductive development stages from R_6 to R_8 according to Fehr and Caviness (*15*). This scale determines the growth stages of soybean seeds, considering the leaf, flower, pod, and seed development. Six samples, each of approximately 2 kg, were harvested at regular time intervals of 4 or 5 days starting at day 101 until seeds had reached physiological maturity (R_8) at 123 days after planting. Harvested samples were divided into three groups: one group was analyzed immediately after harvest, and the second and third groups were dried at 40 and 60 °C, respectively, for 1−4 days, depending upon the initial moisture contents of the seeds, and analyzed afterward. Drying was performed in a ventilated oven, with the seeds still in their pods, awaiting constant weight. Dry seeds were removed from their pods by hand and stored at −20 °C in closed plastic bags until analysis.

Moisture Determination. A total of 2 g of soybean seeds in triplicate were dried in an oven at 105 °C until stable weight and moisture was determined by the difference in weight before and after drying (*16*).

Extraction of Chlorophylls and Greenish Chlorophyll Derivatives. Approximately 5 g of soybeans were ground in a laboratory mill (Polymix KCH-Analytical mill A-10, Kinematika, AG, Luzern, Switzerland). Immediately, a triplicate of 0.5 g were homogenized with 2 mL of cold dimethylformamide p.a. (DMF) and centrifuged at 10000*g* for 10 min. The pellet was suspended again in 2 mL of DMF and centrifuged, to obtain a complete pigment extraction. The supernatants of the two centrifugations were combined and completed with DMF to a final volume of 5 mL. An aliquot was filtered through a 0.22 μm membrane and analyzed by reverse-phased high-performance liquid chromatography (HPLC). All proceedings were realized rapidly, protected from light to reduce enzymatic activity. The use of DMF has been indicated for chlorophyll extraction from samples with high lipid contents and extracted the pigments in a more efficient manner than the commonly used 80% acetone (*17*).

Separation of Chlorophylls and Greenish Chlorophyll Derivatives by RP-HPLC. Analysis was performed according to Sinnecker et al. (*18*), originally described by Mangos and Berger (*19*), with few adaptations regarding the gradient elution and column used. Briefly, the analysis was carried out with a Shimadzu chromatograph (Shimadzu, Tokyo, Japan CLASS-M10A), equipped with a ternary pump solvent delivery and a diode array spectrophotometric detector (SPD-M10AVP) set at 410, 432, and 669 nm, for pheophytins, chlorophylls, and all catabolites simultaneously. Additionally, UV−vis absorption spectra were recorded between 400 and 700 nm. A Shim-pack column (VP-ODS, 5 μm, 250 × 4.6 mm i.d.) with a Thermoquest precolumn (11 × 4.6 mm) and a 1 mL/min solvent flow was employed. The eluents were (A) methanol, (B) 1 M ammonium acetate, and (C) acetone. The following ternary gradient system was employed: After initial conditions (80:20:0, v/v/v), a linear gradient until 15 min (80:0:20) followed by an isocratic hold until 17.5 min (80:0:20) was applied. Another linear gradient changed conditions until reaching 30 min (0:0:100) and later to 34 min (80:20:0). At 40 min, initial conditions were recovered. The isolated pigments were identified by a comparison of absorption spectra and retention times to those of chlorophyll *a* and *b* standards (Sigma Chemical Co., St. Louis, MO). Standard pheophytins *a* and *b* were prepared by acidification of pure chlorophylls *a* and *b* with a few drops of 1 M HCl (*20*).

Quantitative Analysis of Chlorophylls and Greenish Chlorophyll Derivatives. Calibration curves of standards of chlorophylls *a* and *b* were prepared over the range of 0.001−0.04 and 0.001−0.015 mg/mL, respectively. For pheophytins *a* and *b*, calibration curves were prepared within the range of 0.001−0.04 and 0.0002−0.003 mg/mL, respectively. The calibration curves were linear with a correlation coefficient higher than 0.9948.

Extraction of Colorless Chlorophyll Derivatives. The extraction of colorless chlorophyll catabolites was adapted from the methods described by various authors (*21*−*23*). To obtain an extract free of chlorophylls and colored chlorophyll derivatives, 2 g of previously freeze-dried soybean flour (Supermodulyo, Edwards High Vacuum International, Crawley, U.K.) was homogenized with 10 mL of 100 mM methanol/potassium phosphate buffer (1:1, v/v) at pH 7.0, in triplicate, shaken severely, and subsequently centrifuged at 13000*g* for 10 min. Freeze drying was included to enhance the concentration of NCCs, especially in fresh samples, and to start from similar dry weights for extraction. The supernatants were then transferred to clean tubes and centrifuged again. Supernatants of each sample were combined and dried under vacuum. The residues were dissolved in 1 mL of milli-Q H_2O, and the solutions were filtered through 0.45 μm membranes. A 50 μL aliquot containing the colorless chlorophyll derivatives was analyzed by HPLC immediately after extraction. All proceedings were performed at minimum time to reduce exposure to light and to minimize eventual enzymatic degradation.

Separation of Colorless Chlorophyll Derivatives by RP-HPLC. Analysis was performed on the basis of former publications (*24*, *25*). The HPLC system used was the same as described before. A 0.5 mL/min flow rate was employed; absorbance was monitored at 459, 320, and 210 nm; and the spectra were recorded between 190 and 800 nm. Sample injection was 50 μL, and a ternary gradient system consisting of (A) water, (B) 100 mM K_3PO_4 buffer at pH 7.0, and (C) methanol was used. The gradient was developed as follows: Initial conditions (0:80:20, v/v/v) were held until 10 min, followed by a linear gradient to 70 min (0:40:60), which was held until 80 min. Afterward, conditions were changed linearly to 82 min (20:20:60) and then to 87 min (20:10:70), 90 min (15:5:80), 96 min (9:1:90), 97 min (4.5:0.5:95), 98 min (5:0:95), and 100 min (0:0:100). These conditions were held until 115 min, then changing to 120 min (80:0:20), and held until 125 min. At last, a linear gradient led to initial conditions at 135 min. All analyses were performed in triplicate with the same dry weight for all samples. Relative NCC concentrations were expressed as areas under the curve because of the lack of standards.

Statistical Analysis. Obtained data are presented as mean values of analyses in triplicate. All triplicates had a variation coefficient lower than 5%. Differences between data were submitted to a Tukey test, with a confidence interval of 0.95 (STATGRAPHICS, version 2.6).

RESULTS AND DISCUSSION

Analyses of Greenish Chlorophyll Catabolites. Figure 1 presents the contents of chlorophylls and pheophytins analyzed by HPLC. In freshly harvested samples, chlorophylls *a* and *b* were the only pigments found in significant amounts over the whole maturation period. Contents of chlorophylls *a* and *b* at the first point of harvest (101 days after plantation, correspond-

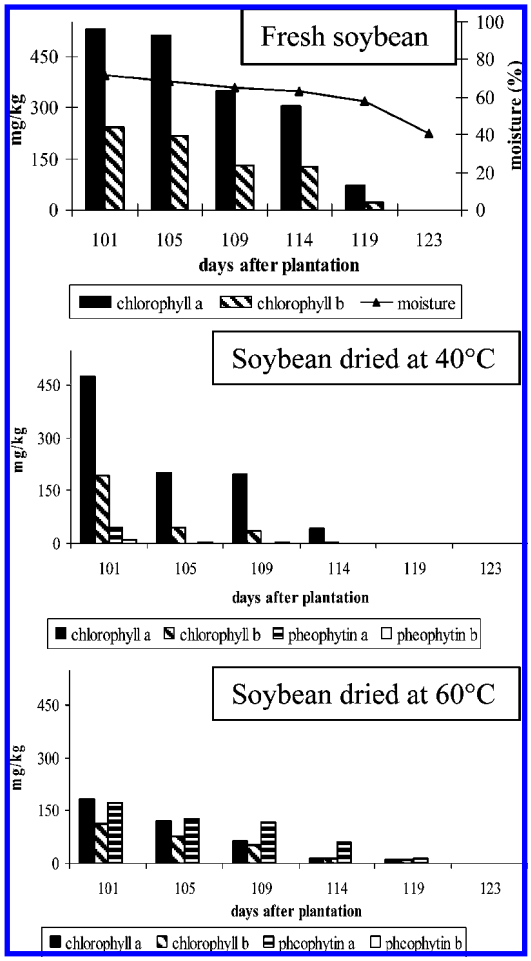

Figure 1. Chlorophyll and pheophytin contents during maturation of fresh soybean seeds IAC-18 and influence of postharvest drying at 40 and 60 °C.

ing to the stage R_6) were very high, 527.7 ± 21.6 and 243.4 ± 11.1 mg/kg, respectively, because seeds had not reached physiological maturity yet. At the second point (105 days), the amounts were still similar, and only from this point on, chlorophyll contents declined rapidly. Colored degradation products, such as chlorophyllides, pheophytins, and pheophorbides, were not detected and might have been formed and further degraded immediately to colorless derivatives, suggesting a series of degradation reactions. As expected, moisture contents were high at premature harvests and allowed enzymes to degrade chlorophylls until the seeds achieved physiological maturity (R_8) at 123 days after planting. Literature shows that, from R_8 up and during the next 2 weeks, moisture decreases drastically, enzyme activity stops, and seeds achieve commercial maturity, although final moisture and green pigment contents are highly dependent upon climate conditions (*15*).

Samples harvested over the whole maturation period and then dried at 40 °C before HPLC analysis had a similar degradation profile when compared to freshly harvested samples, but amounts of chlorophylls *a* and *b* were slightly lower (476.9 ± 22.2 and 192.6 ± 2.2 mg/kg, respectively) at the first time of harvest. At the same time, trace amounts of 47.0 ± 2.3 mg/kg of pheophytin *a* and 9.9 ± 0.3 mg/kg of pheophytin *b* were detected but were reduced to zero in later stages. This indicates that pheophytinization had taken place during the drying process at this temperature, in addition to enzyme-mediated degradation.

Samples dried at 60 °C showed a significant loss of pigments and, at the first harvest, contained only 183.9 ± 7.7 and 111.9 ± 3.7 mg/kg of chlorophylls *a* and *b*, respectively. This seems to be the result of the susceptibility of chlorophylls to high temperature, acid environment, oxygen, light, and subsequent degradation to other substances (*19*). Nevertheless, under this condition, the proportion between pheophytins *a* and *b* changed from 2.5 at 40 °C to relatively high amounts of pheophytin *a* (172.0 ± 8.5 mg/kg) and trace amounts of pheophytin *b* (0.9 ± 0.05 mg/kg). This might be due to a higher susceptibility of pheophytin *b* to further degradation than pheophytin *a* at high temperatures (*26*). The sum of chlorophylls and pheophytins was 468.7 mg/kg and corresponds to 60.8% of chlorophyll contents in fresh samples.

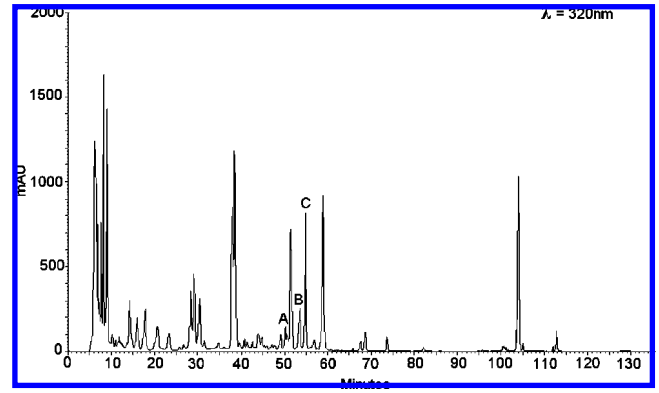

Figure 2. Analytical HPLC chromatogram recorded at $\lambda = 320$ nm of a fresh NCC extract of soybean IAC-18, at 114 days after harvest. Tentatively identified nonfluorescent catabolite peaks were named A, B, and C.

All analyzed samples contained more chlorophyll *a* and pheophytin *a* than chlorophyll *b* and pheophytin *b*, respectively, and all pigments suffered a rapid reduction during the ripening process. From the fifth harvest date on (119 days after plantation), the amounts of all green pigments were close to zero, thus far in fresh as in dried samples.

Analysis of Colorless Chlorophyll Metabolites. The NCCs are the first linear tetrapyrrolic intermediates during chlorophyll breakdown, in which the four pyrrolic units are completely deconjugated and that contain an α-formyl-pyrrol group at ring B of the tetrapyrrol (*27*). Therefore, the provisional identification of the NCCs was made by searching for a maximum absorption at 320 nm with no shoulder that stands for the formyl-pyrrol group. **Figure 2** provides the chromatogram of one of the samples analyzed by reverse-phase HPLC between the ripening stages R_6 and R_8. All samples showed complex chromatograms, but three peaks named A, B, and C, according to their retention times, were selected possessing UV/vis spectra with characteristics considered typical for NCCs (*25*). Peak C was predominant, but the other two peaks showed the same spectral characteristics and behavior. The other peaks in the chromatograms correspond to a large number of unknown substances, with an absorption maximum at 330 nm. Because solvent extractions and HPLC analyses were based on the same sample dry weight, it was possible to express the relative concentrations as areas under the curve, because there are no commercially available standards for NCCs.

Figure 3 shows the absorption spectra of the fractions A, B, and C. The main reason for considering the isolated peaks as NCCs was the presence of an absorption maximum at 320 nm that stands for the formyl-pyrrol moiety. Although the presence of an absorption maximum at 320 nm has been considered a characteristic of NCCs, published UV/vis spectra differ slightly in localization and intensity of the maximum absorbance depending upon the plant species and parts analyzed. Therefore, the observed absorption maxima in our work are slightly

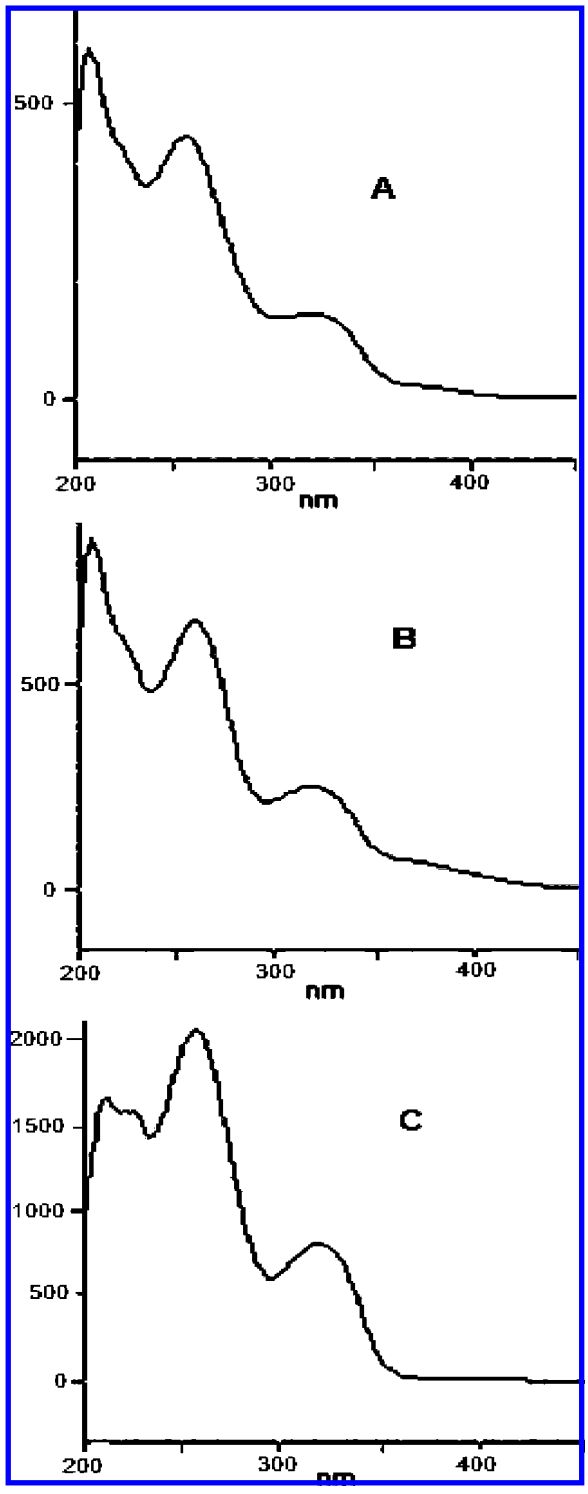

Figure 3. UV/vis absorption spectra of the nonfluorescent chlorophyll catabolites recorded during analytical HPLC of soybean extracts. Letters A, B, and C indicate the fractions identified as NCCs.

different from those formerly published for spinach, *Arabidopsis thaliana*, maize leaves, and pears (*13*, *25*, *28*, *29*). For example, about 15 different NCCs have been found in senescent leaves. Thus, we cannot prove that we have found the same NCCs as reported, but maybe they are new NCCs, specific for soybean with slight structural modifications. To our knowledge, there are no data on NCCs in soybeans up to now.

In **Figure 4**, the mean areas under the curves of these fractions analyzed in triplicate are shown, corresponding to fresh soybean as well as soybean dried at 40 and 60 °C over the whole

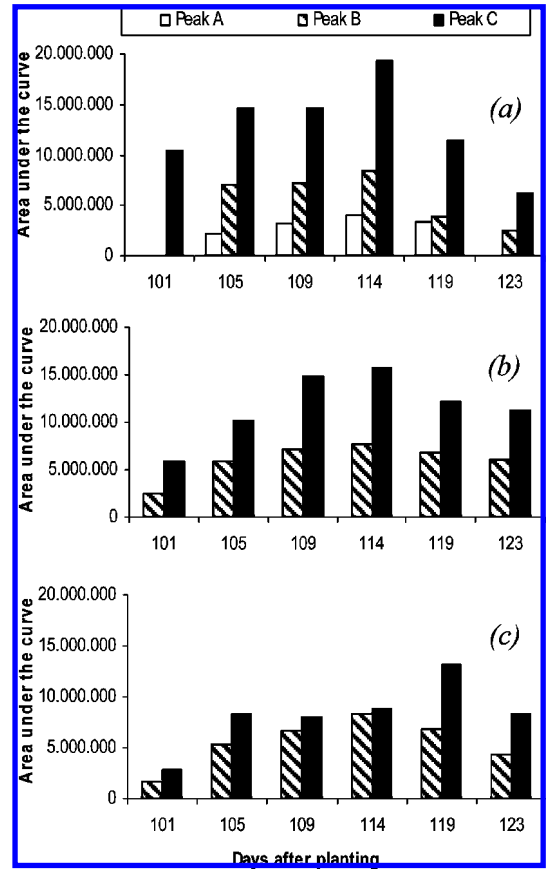

Figure 4. (a−c) Mean areas under the curve of the fractions identified as NCCs during natural maturation of soybean IAC-18. The letters A, B, and C correspond to the fractions A, B, and C, respectively: (a) fresh soybeans, (b) soybeans dried at 40 °C, and (c) soybeans dried at 60 °C.

maturation period. Their relative amounts increased until the fourth harvest point (114th day after planting), because of high initial amounts of chlorophyll that were constantly degraded. After the fourth harvest point, the NCC diminished, because there was few chlorophyll left to form NCCs and the NCCs themselves were further degraded or suffered further modification.

In the samples dried at 40 °C, lower amounts of NCCs were observed but the profile of appearance and disappearance of NCCs was similar to that in the fresh samples. Peak A was not identified probably because of its low concentration.

In the samples dried at 60 °C, the very low amounts of chlorophylls together with the fact that enzymes had been partially inactivated resulted in retardation of NCC formation as well as lower areas of NCCs under the curves. Our results agree with the hypothesis of Kräutler (*27*) and the revision of Takamiya, Tsuchiya, and Ohta (*30*), who proposed a possible degradation of NCCs to monopyrrolic structures in senescent leaves and cotyledons of different plants.

In conclusion, chlorophyll degradation is apparently accelerated by postharvest drying at temperatures above 40 °C, showing lower chlorophyll levels with raising drying temperatures. Because of the high drying temperatures, it is assumed that the chlorophyll degradation is not catalyzed by enzymes, which are partially or fully inactivated, but that there is a temperature-dependent pheophytinization in an acidic medium. The NCC concentration rose until 114 days after plantation, because of constant degradation of chlorophyll, and then decreased as chlorophyll levels became lower. Consequently, less NCCs were produced. Therefore, our results support the hypothesis that there

is no accumulation of NCCs in ripening soybean seeds but, instead, that NCCs are further degraded, probably to form other structures important for germination.

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