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Changes in Polyphenols of the Seed Coat during the After-Darkening Process in Pinto Beans (*Phaseolus vulgaris* L.)

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Proanthocyanidins and flavonoids were isolated and identified from seed coats of two aged and nonaged pinto bean lines: 1533-15 and CDC Pintium. The seed coat of 1533-15 darkens slowly and never darkens to the same extent as CDC Pintium. Analysis of the overall level of proanthocyanidins using a vanillin assay demonstrated that aged and nonaged seed coats of CDC Pintium had significantly higher levels of proanthocyanidins than aged and nonaged 1533-15 seed coats. Aged and nonaged seed coats of both lines were found to contain one main flavonol monomer, kaempferol, and three minor flavonols, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-glucosylxylose, and kaempferol 3-*O*-acetylglucoside. These compounds were identified by NMR and ESI-MS analysis (except for kaempferol 3-*O*-acetylglucoside, which was tentatively identified only by ESI-MS analysis) and quantified using HPLC-DAD. The combined concentrations of all the kaempferol compounds in seed coats of CDC Pintium were significantly higher than in seed coats of 1533-15, and the combined contents did not change after aging. The content of kaempferol decreased nearly by half in the seed coats of CDC Pintium after aging, whereas no significant change was observed in the seed coats of 1533-15. Proanthocyanidin fractions from both lines, aged and nonaged, were subjected to LC−MS/MS analysis and found to be composed primarily of procyanidins. Procyanidins in the seed coats were predominantly polymers with the degree of polymers higher than 10. The proportion of these polymers decreased after aging, while that of the low-molecular-weight procyanidins increased. A catechin−kaempferol adduct was tentatively identified in both lines by LC−MS/MS, and the concentration increased in the seed coats after aging.

KEYWORDS: ***Phaseolus vulgaris*; seed coat darkening; polyphenols, flavonols**

INTRODUCTION

Consumers have developed specific preferences for seed size, shape, and color in dry bean (*Phaseolus vulgaris* L., Fabaceae) (*1*), and these preferences determine the various market classes. Postharvest darkening, or after-darkening, of the seed coat is a phenomenon that occurs in some market classes of dry bean, and is a particular problem in pintos and reds. It causes considerable loss in value because of a decline in visual quality as preferred by consumers and an associated increase in cooking time (*2*). Seed coat darkening and increased cooking time are associated with the "hard-to-cook" (HTC) phenomenon found in some legume species, particularly dry bean. The exact causes of after-darkening are not well-known, but they seem to include a combination of environment, genetics, and chemical changes that take place within the seed coat. Darkening is accelerated by exposure to light, high temperature, and humidity during storage (*3*, *4*).

Breeding lines of pinto beans that darken considerably more slowly than current varieties have been developed by the dry bean breeding program at the University of Saskatchewan. Preliminary genetic analysis suggests that the slow-darkening trait is simply inherited. The presence of the recessive allele of the bean seed coat color gene *J* (syn. *L*) has been associated with reduced levels of postharvest darkening (*5*−*7*), and chemical analyses have associated this gene with proanthocyanidin production in yellow beans (*14*). It has also been shown that seed coat phenols and polyphenols are responsible, at least in part, for the after-darkening and HTC phenomenon (*8*, *9*).

As part of our investigation of the after-darkening process, we sought to chemically characterize the seed coat compounds present in a slow-darkening pinto breeding line (1533-15) and a pinto bean cultivar (CDC Pintium) that after-darkens more rapidly. Knowledge of the types and amounts of phenolic compounds responsible for after-darkening could assist in the

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identification of the genes responsible as well as the mechanism by which seed coats darken.

MATERIALS AND METHODS

Seed Material. CDC Pintium is an early-maturing, rapid-darkening pinto bean cultivar released by the University of Saskatchewan and currently grown in western Canada. 1533-15 is an F_4-derived line from the cross CDC Pintium × SC11743-3, and was selected for slow-darkening and adaptation to Saskatchewan growing conditions. Seed of both 1533-15 and CDC Pintium was grown in the field near Saskatoon, SK, in 2003. Half of the harvested seed was aged by placing samples in plastic bags to maintain humidity and then exposing them to light for two months by laying them on benches in a greenhouse. Average daily accumulated light levels measured in the greenhouse during this January to March period were approximately 3.84 mol m^{-2} d^{-1} photosynthetic photon flux. Temperatures in the greenhouse were between 16 °C (night) and 24 °C (day). The bags were shaken and rotated weekly during aging to ensure the seeds received equal exposure to light. Nonaged seed was stored in sealed bags in a cold room (4−10 °C, lights off) to preserve freshness until analysis.

Tissue Preparation for Extraction of Phenolics. Seed from both lines was soaked in distilled water, and the seed coats were manually decorticated. The seed coats were then frozen at −80 °C, freeze-dried, and ground to a fine powder in a Braun coffee grinder, and 3−4 g was loaded into glass chromatography columns (26.0 × 1.5 cm) and eluted with 100 mL of acetone/water/acetic acid (70:29.5:0.5). This procedure was performed nine times for 1533-15 and six times for CDC Pintium until a total of 52.64 g of 1533-15 seed coats and 22.7 g of CDC Pintium seed coats had been extracted.

Isolation of Flavonols. Extracts obtained above were rotary evaporated to dryness, redissolved in 3.0 mL of MeOH/H_2O (1:1, v/v), placed under N_2 to evaporate the MeOH, and then freeze-dried. Extracts were added to 3.0 mL of MeOH/H_2O (8:2, v/v), sonicated for 30 min to dissolve, then loaded into glass chromatography columns (26.0 × 1.5 cm) containing 5.0 g of Sephadex LH-20 (Sigma-Aldrich Co.) which had been equilibrated with 50 mL of MeOH/H_2O (8:2, v/v), eluted with 50 mL of MeOH/H_2O (7:3, v/v), and separated by gravity feed. The compounds were monitored every 10 min as they came off the column with a hand-held UV detector set at 366 nm. The flavonoid fractions appeared brown under UV light.

Since HPLC analysis demonstrated two fractions that consisted mainly of flavonols, they were combined, rotary evaporated, and reisolated prior to further analyses. The samples were taken up in 1.0 mL of 100% MeOH, and initially 10 μL injections were made, gradually increasing to 100 μL. Individual peaks from each injection were collected in glass scintillation vials and dried under a flow of N_2. This final purification of flavonols from four HPLC peaks was achieved on an Agilent model 1100 HPLC instrument (Agilent Technologies Canada Inc., Mississauga, ON, Canada) equipped with a degasser, quad pump, autosampler, and diode array detector (DAD). Samples were scanned (200−550 nm) with a stored wavelength of 264 and 366 nm. The column used was a CapCell Pak AG 120 C18 (Shiseido Co. Ltd., Japan; 4.6 × 250 mm, 5 μm particle size), and the HPLC instrument had ChemStation 3.0 software. The solvent system used was the same as that used by Abou-Zaid et al. (*10*) and was as follows: solvent A = 5% aqueous formic acid; solvent B = acetonitrile/methanol (5:95); elution steps, 0−3 min, isocratic (85% A/15% B); 3−8 min, gradient (85% A/15% B to 76% A/24% B); 8−11 min, isocratic (76% A/24% B); 11−18 min, gradient (76% A/24% B to 66% A/34% B); 18−28 min, gradient (66% A/34% B to 54% A/46% B); 28−39 min, gradient (54% A/46%B to 5% A/95% B); 39−42 min, isocratic (5% A/95% B); 42−47 min, gradient (5% A/95% B to 85% A/15% B); 47−55min, isocratic (85% A/15% B). The flow rate was set to 0.9 mL/min.

Individual peaks were collected numerous times and dried under N_2 until enough material of each compound was collected for ^1H and ^{13}C NMR analysis. Purity of the compounds prior to NMR analysis was achieved by reinjection of collected peaks and verification of the presence of a single peak for each compound, which was then identified as pure using the UV purity index of the Chemstation 3.0 software.

NMR Analyses. To prepare samples, 8.6 mg of compound **1**, 3.2 mg of compound **2**, 4.5 mg of compound **3**, and 3.8 mg of compound **4** were each dissolved in 1.0 mL of deuterated dimethyl sulfoxide (DMSO-d_6) and placed in NMR tubes. Spectra were obtained with a Bruker AV 600 MHz spectrometer (Bruker AG, Switzerland). 1D ^1H and ^{13}C NMR as well as 2D DQF-COSY, HSQC, and HMBC analyses were conducted.

Quantification of Flavonols by HPLC-DAD Analysis. Each of the compounds **1**−**4** was made up to concentrations of 0.125, 0.0625, 0.03125, and 0.01563 mg/mL, and standard curves were constructed. Kaempferol (**1**) was purchased from Sigma Co., whereas the other compounds (**2**−**4**) were those isolated from the CapCell Pak C_{18} column and identified by NMR and ESI-MS. Injections of 25 μL of the standards were made, whereas 100 μL injections were made of the samples. Samples for HPLC analysis were prepared as follows: the seed coat material was freeze-dried and then ground to a fine powder with three glass beads per sample by shaking in a paint shaker for 12 min on high speed. Ethanol was added, and the samples were again shaken for 12 min. The samples were centrifuged for 10 min at 14000 rpm using an Eppendorf centrifuge 5415C (Brinkman Instruments Inc., Westbury, NY). The ethanol was removed by using a DNA 110 Speed Vac (Savant Instruments Inc., Holbrook, NY). Three replications of three seeds each were used for each bean line. Quantitation of the flavonols was achieved on a Varian Prostar HPLC instrument (Varian Inc., Palo Alto, CA) with a model 230 solvent delivery module, 410 autosampler, and 330 photodiode array detector, and the wavelength used for quantification was 350 nm. The column used was a CapCell Pak AG 120 C18 (Shiseido Co. Ltd., Japan; 4.6 × 250 mm, 5 μm particle size), and the HPLC instrument had Galaxy software. The solvent system used was the same as described in ref *10* and given above.

ESI-MS Analyses of Flavonols. Purified samples of compounds **2**−**4** were analyzed on a Micromass Quatro II triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.). The samples were acquired in negative ion mode (cone voltage 30 V) using flow injection analysis in methanol/water (1:1, v/v) via an Agilent 1100 HPLC system. The data range was m/z 80−1000, the source temperature was 80 °C, and the desolvation temperature was 200 °C.

Vanillin Assay To Determine Total Proanthocyanidin Concentrations. Five replications of three seeds each were taken from aged and nonaged samples of each of CDC Pintium and 1533-15. The seeds were weighed, soaked in a minimal amount of deionized water, and manually decorticated. The seed coats were then freeze-dried and ground to a fine powder in Eppendorf tubes using a Retsch MM 300 mixer mill (Retsch GmbH, Haan, Germany) for 15 min without solvent. Then 1.5 mL of acetone/H_2O (7:3, v/v) was added, and the seed coats were subjected to another 15 min of homogenization. The samples were then centrifuged for 5.0 min at 5000 rpm, and the supernatant was drawn off. The supernatant was dried by rotary evaporation and taken up in 1.0 mL of 95% ethanol. The vanillin−HCl assay (*11*) was carried out using a solution of 2% vanillin−HCl and (±)-catechin (Sigma-Aldrich Co.) to construct the standard curve.

Isolation of Proanthocyanidins. Dried seed coats were extracted with acetone/water/acetic acid (70:29.5,0.5 v/v), loaded onto a 26.0 × 1.5 cm glass chromatography column containing 5.0 g of Sephadex LH-20 gel which had been equilibrated with 50 mL of MeOH/H_2O (8:2, v/v), eluted with 50 mL of MeOH/H_2O (7:3, v/v), and separated by gravity feed. After this elution, the solvent ran clear in color but the Sephadex column retained a brown color under visible light, which is characteristic of proanthocyanidins remaining on the column (*12*). The proanthocyanidins were then eluted with 50 mL of 50% aqueous acetone, dried by rotary evaporation, taken up again in 2.0 mL of 100% MeOH, and placed in glass scintillation vials. These were then dried under a stream of N_2, and 148.5 mg of CDC Pintium extract and 57.6 mg of 1533-15 extract were then used for LC−MS/MS analysis.

LC−MS/MS Analyses of Proanthocyanidins. Chromatographic separation was performed on an Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) consisting of a binary pump, a quaternary pump, a solvent degasser, an autosampler, a thermostat column compartment, a diode array detector, and a fluorescence detector. Separation was carried out on a 250 × 4.6 mm Phenomenex

Table 1. Concentrations of Flavonols from Seed Coats of Pinto Bean Lines 1533-15 and CDC Pintium[a]

	concn (mg/g of dry weight of seed coat)			
	1533-15, nonaged	1533-15, aged	Pintium, nonaged	Pintium, aged
kaempferol	0.613 b (0.084)	0.520 b (0.022)	1.406 a (0.141)	0.842 b (0.208)
kaempferol 3-*O*-glucosylxylose	0.021 b (0.007)	0.010 b (0.002)	0.079 a (0.005)	0.098 a (0.021)
kaempferol 3-*O*-glucoside	0.075 b (0.021)	0.054 b (0.062)	0.198 b (0.079)	0.492 a (0.012)
kaempferol 3-*O*-acetylglucoside	0.037 c (0.007)	0.051 c (0.009)	0.117 b (0.005)	0.146 a (0.009)
total flavonols	0.747 a (0.074)	0.636 a (0.028)	1.800 b (0.227)	1.577 b (0.1910)

[a] Means are given with standard errors in parentheses. Means followed by the same letter within a row are not significantly different ($P > 0.05$), $n = 3$.

Figure 1. Seed of CDC Pintium (top) and 1533-15 (bottom) before (left) and after (right) aging.

Luna Silica (2) column (Phenomenex, Torrance, CA) with a particle size of 5 μm at a column temperature of 37 °C. The tertiary mobile phase consisted of (A) methylene chloride, (B) methanol, and (C) acetic acid and water (1:1, v/v). The 70 min gradient was 0−20 min, 14.0−23.6% B, linear; 20−50 min, 23.6−35.0% B, linear; 50−55 min, 35.0−86.0% B, linear; 55−65 min, 86.0% B, isocratic; 65−70 min, 86.0−14.0% B, linear, followed by 10 min of reequilibration of the column before the next run. A constant 4.0% C was kept throughout the gradient. UV detection was recorded at 280 nm (8 nm bandwidth) versus a reference wavelength at 650 nm (30 nm bandwidth). For fluorescence detection, the excitation and emission wavelengths were 276 and 316 nm, respectively.

The eluting stream (1 mL/min) from the HPLC apparatus was introduced into a Bruker Esquire-LC ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Enhancement of ionization of procyanidins using a postcolumn addition of 10 mmol/L ammonium acetate has been described previously for beans and other foods (*13*).

Statistical Analyses. Statistical analyses were carried out on proanthocyanidin and flavonol data using SAS 7.0 (SAS Institute, Cary, NC). One-way analyses of variances (ANOVAs) were carried out using the General Linear Model (GLM) procedure. Significant differences between the means were determined using the Student−Newman−Keuls test. Total flavonol concentrations were analyzed using the means of individual flavonols (**Table 1**).

RESULTS AND DISCUSSION

Seed Samples. Seed samples aged in the greenhouse darkened more than the samples stored in the cold room. Samples of 1533-15 were considerably lighter than those of CDC Pintium after aging. **Figure 1** shows what seeds of the two lines look like before and after aging.

Flavonol Composition in Aged and Nonaged Pinto Bean Lines. Several crude extracts had to be made for each seed coat sample to obtain sufficient amounts for analysis. Typically, a 3.73 g sample of CDC Pintium seed coats yielded 482.3 mg of freeze-dried material while a 3.23 g sample of 1533-5 seed coats yielded 300.3 mg of freeze-dried material.

HPLC analysis of the unfractionated crude acetone/water extract of the seed coats of 1533-15 yielded a chromatogram containing four peaks (**Figure 2**). There was a substantially higher concentration of one compound than the other three. After Sephadex LH20 fractionation and HPLC verification of this material, two of the four fractions were shown to contain flavonols. One fraction contained a single compound (compound **1**) at high concentration, while the other was found to contain three compounds (compounds **2**−**4**). These same four compounds were found to be in the CDC Pintium crude extracts as well. Once these compounds were purified by HPLC, their structures were elucidated using NMR and ESI-MS. Comparing the NMR spectra obtained for compounds **1**−**3** with published spectra (*14*−*17*), these compounds were determined to be

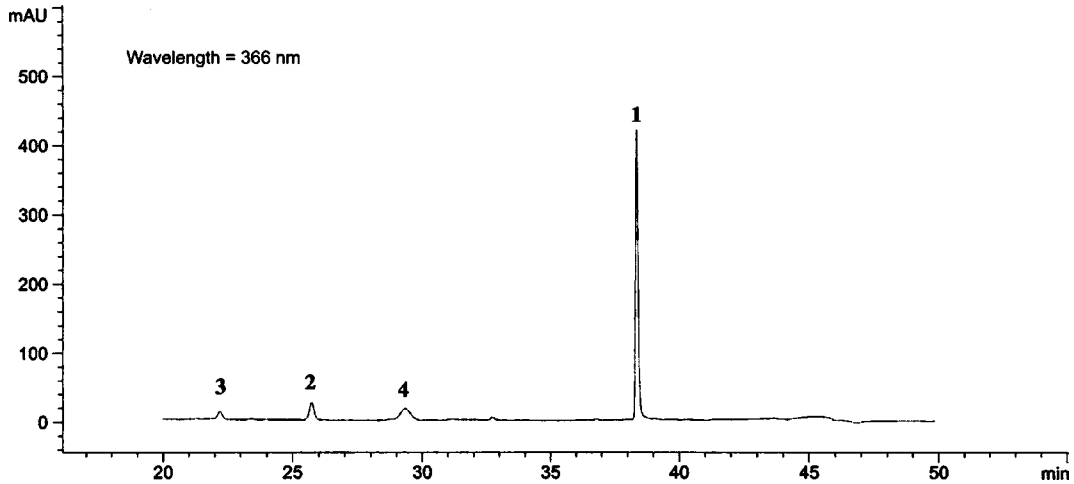

Figure 2. HPLC trace of a 10 μL injection of a crude unfractionated extract (1.0 mg/mL) obtained from pinto bean line 1533-15 showing compounds **1**–**4**.

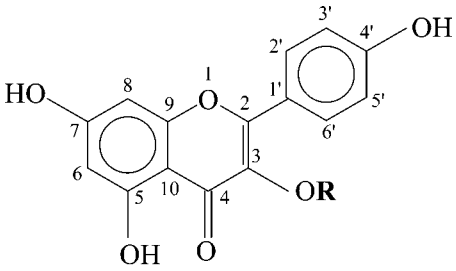

Figure 3. Chemical structures of flavonoids (compounds **1**–**4)** found in pinto beans based on NMR and ESI-MS data.

kaempferol, kaempferol 3-*O*-*β*-glucosyl-*β*-xylose, and kaempferol 3-*O*-*β*-glucoside, respectively. Unfortunately, compound **4** was found to be labile and degraded in the NMR solvent, so its identity is tentative and based solely on the ESI-MS data. ESI-MS analysis was not used for the identification of kaempferol (compound **1)** since its structure was readily determined from the NMR data. The ESI-MS analysis of compounds **2**−**4** gave monoisotopic molecular ion peaks $[M - H]^-$ at *m*/*z* 579.08, 447.04, and 489.04 corresponding to kaempferol 3-*O*-glucose-xylose, kaempferol 3-*O*-glucoside, and kaempferol 3-*O*-acetyl-glucoside. Structures for the four compounds are given in **Figure 3**. Kaempferol 3-*O*-acetylglucoside (compound **4**) has previously been identified in *P. vulgaris* on the basis of MS data (*18*).

The results of quantifying these flavonols in aged and nonaged pinto beans of 1533-15 and CDC Pintium by HPLC are illustrated in **Table 1**. Nonaged CDC Pintium had significantly higher levels of the main flavonoid, kaempferol, than both aged and nonaged 1533-15 ($P < 0.05$). Interestingly, aged CDC Pintium had significantly lower levels of kaempferol than nonaged CDC Pintium, and the amounts did not differ significantly from those of both aged and nonaged 1533-15 (**Table 1**). It is uncertain why the CDC Pintium seed coats appear to lose kaempferol with aging; however, LC−MS/MS analysis (described below) has shown that there are kaempferol−catechin adducts which form in both lines. Perhaps the kaempferol is combining with proanthocyanidin monomers and polymers over time, and thus is not available for HPLC quantitation. Since CDC Pintium initially has more kaempferol and proanthocyanidins than 1533-5, there is more available to combine as the seed coats age. There does not appear to be any pattern of loss with time for the other flavonol glycosides present in the seed coat of the two lines. In fact, there is an increase over time in the glycosylated flavonols in CDC Pintium (**Table 1**). To try to gain a clearer picture of the total concentration of flavonols in both types of bean lines as a function of aging, all of the flavonol data were combined (see the Materials and Methods) and are given in **Table 1**. CDC Pintium, both aged and nonaged, had 2-fold higher total flavonols compared with the 1533-15 samples, and this difference was highly significant. However, neither 1533-15 nor CDC Pintium showed significant losses in total flavonols with time.

Proanthocyanidins in Aged and Nonaged Pinto Beans. In the vanillin assay for proanthocyanidins (which detects flavan-3-ol monomers as well as flavan-3-ol polymers), samples of aged and nonaged CDC Pintium seed coats had approximately 3 times the concentration of proanthocyanidin as compared to 1533-15 (**Figure 4**). After aging, neither line showed a significant reduction in the amount of total proanthocyanidins. To date, relatively little is known of how seed coat polyphenols

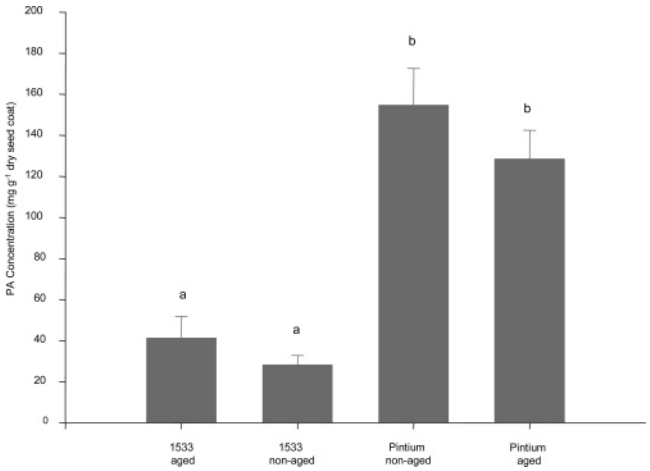

Figure 4. Total proanthocyanidin concentration of aged and nonaged 1533-15 and CDC Pintium based on a vanillin assay. Proanthocyanidin concentrations are expressed as (±)-catechin equivalents. Bars with the same letters are not statistically different at $P < 0.05$.

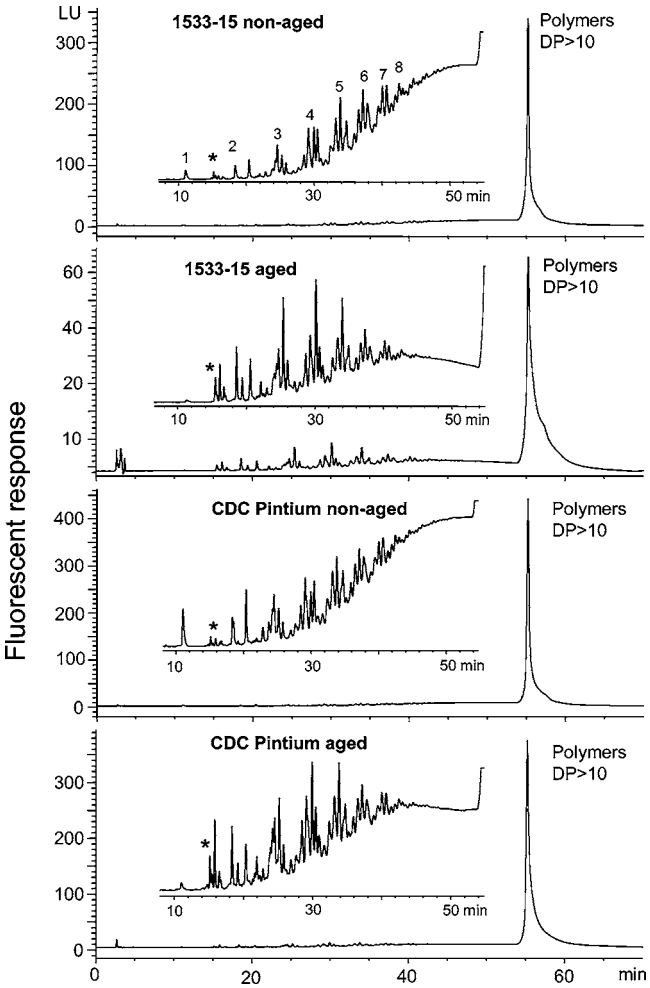

Figure 5. Normal-phase HPLC fluorescent profiles of proanthocyanidins in aged and nonaged samples of two pinto bean lines (CDC Pintium and 1533-15). Numbers on the peaks denote the degree of polymerization of proanthocyanidins in the peak. The asterisk denotes an adduct dimer (catechin–kaempferol).

change with time, but oxidation of proanthocyanidins is suspected as one cause of seed coat darkening. For example, it is known that grape (*Vitis vinifera* L. cv. Pinot Noir) skin and

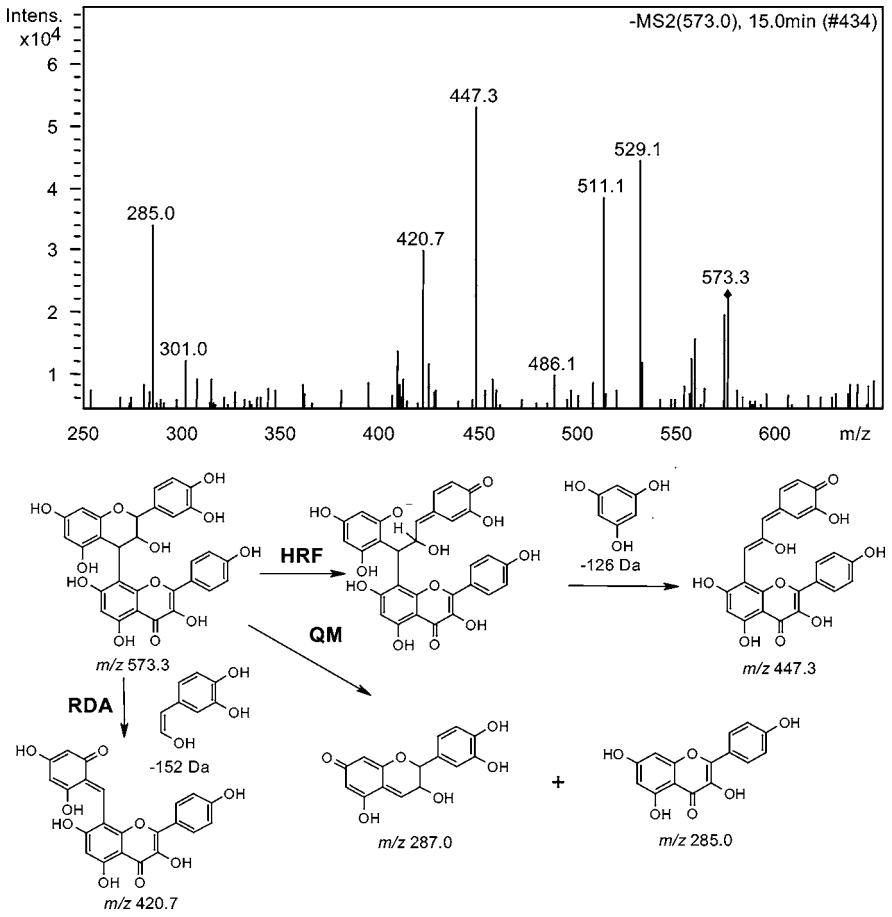

Figure 6. Product ion spectrum of the catechin–kaempferol dimer recovered from the proanthocyanidin sample and the proposed fragmentation pathway. The fragment mechanisms are RDA (retro-Diels–Alder), HRF (heterocyclic ring fission), and QM (quinone methide).

seed proanthocyanidins will degrade under basic conditions and under exposure to atmospheric oxygen (*19*).

To understand the distribution of oligomers and polymers of proanthocyanidins in pinto bean and possible structural changes after aging, proanthocyanidins extracted from pinto bean coats were analyzed with normal-phase HPLC with tandem mass spectrometry detection. The representative fluorescent profiles are shown in **Figure 5**. Mass spectrometry analysis indicated that proanthocyanidins in the seed coats were predominantly procyanidins with catechin or epicatechin as subunits. Propelargonidins with (epi)catechin and (epi)afzelechin were also detected as minor components, contributing to less than 5% of the total proanthocyanidins according to the peak area. The structures of these propelargonidins and procyanidins have been reported previously (*13*). On the basis of peak area, 80.1 ± 3% of the total proanthocyanidins in nonaged 1533-15 seed coats were polymers of greater than 10 subunits and the reminder (19.9 ± 2%) were monomers through decamers. The proportion of monomers through decamers increased significantly to 33.8 ± 2% ($P < 0.05$) in the aged 1533-5 samples, indicating depolymerization during aging. A similar portion of proanthocyanidins in CDC Pintium were monomers through decamers (17.2 ± 1%), and this increased to 21.3 ± 2% ($P < 0.05$) of the total proanthocyanidin after aging.

Formation of Flavan-3-ol−Flavonol Adducts in Aging of Pinto Bean Seed Coats. A catechin−kaempferol dimer adduct was tentatively identified in pinto bean seed coats at 15 min in normal-phase HPLC. The product ion spectrum of this adduct and the proposed fragmentation pathway are shown in **Figure 6**. The major ions containing the structural information were *m*/*z* 447.3, 420.7, and 285.0. The *m*/*z* 420.7 ion is derived from *m*/*z* 573.3 after a 152 Da neutral loss. Elimination of ring B from the flavan-3-ol through retro-Diels−Alder (RDA) reaction of ring C led to this ion. The RDA reaction took place on the top unit of a dimer. Loss of 152 Da indicates that ring B of the top unit has two hydroxyl groups. The *m*/*z* 447.3.0 $[M - H - 126]^-$ ion is formed after the elimination of ring A from the dimer through heterocyclic ring fission (HRF). Loss of 126 Da indicates that ring A of the top unit has a 1,3,5-trihydroxybenzene structure. Thus, the top unit of this dimer is tentatively identified as catechin. Because the chirality of C-3 on the flavan-3-ols cannot be determined by mass spectrometry, the catechin may also be epicatechin. This dimer was also cleaved by quinone methide to produce *m*/*z* 285, a deprotonated kaempferol. The position of the interflavan bond was most likely on C6 or C8 of kaempferol; however, the exact position could not be determined from the mass spectrum. The adduct dimer was detected in both 1533-15 and CDC Pintium. Aging increased the amount of adducts by 3−5-fold on the basis of the peak area. It is most likely these are adducts are formed during the process of oxidation.

In summary, we have identified the main flavonol monomers in seed coats of a slow-darkening pinto bean line (1533-15) and in a cultivar (CDC Pintium) that darkens more rapidly. These compounds have been quantified, and the main flavonoid, kaempferol, was found to decrease significantly with aging in CDC Pintium but not in 1533-15. In addition, we have found that CDC Pintium, both aged and nonaged, contains significantly more total flavonols than does either aged or nonaged 1533-15. Aged and nonaged CDC Pintium seed coats also contain significantly more proanthocyanidins as measured by the vanillin assay than do samples of aged or nonaged 1533-15. The

proanthocyanidins in these two lines (aged and nonaged) are composed of procyanidins, and the degree of polymerization decreases with aging. We have also found that kaempferol−catechin dimers form in the seed coat and that the proportion of these dimers increases as the seed coat ages. In sorghum (*Sorghum bicolor* L. Moench), secondary changes and complexes of proanthocyanidins with other phenolics in the seed coat may result in brown seed coat colors that darken with development (*20*). Red beans also tend to darken with storage, and they have been shown to contain proanthocyanidins, including kaempferol 3-*O*-*β*-D-glucoside (astragalin) (*14*), although this has not yet been associated with the darkening process.

The objective of this study was to identify compounds that may be linked to after-darkening in pinto bean. Making any definitive statements as to the exact causes of after-darkening is difficult based on the analysis of only two contrasting genotypes. Differences shown may simply be due to other genetic differences between the two lines. Since 1533-15 is closely related to CDC Pintium, however, there is a good possibility that the differences seen here are indeed related to after-darkening. To more clearly associate the chemical analyses with the after-darkening phenomenon, the genetics must be linked to the chemistry. This study shows that there are quantitative differences in the amounts of polyphenols between slow- and fast-darkening lines and that flavonols interact with proanthocyanidins during the aging process. Analysis of the levels of these compounds in the segregating progeny of a cross between CDC Pintium and 1533-15 should allow for the association of specific compounds with the nondarkening trait and give a clearer indication of the biochemical processes leading to after-darkening in pinto bean.

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