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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JULY 2010

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ARTICLE

Impact of Postharvest Handling on Carotenoid Concentration and Composition in High-Carotenoid Maize (*Zea mays* L.) Kernels

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High carotenoid maize is an ideal source of high value dietary carotenoids, especially lutein and zeaxanthin, in human and animal feed and has been proposed as a feedstock for high carotenoid egg production. A modified analytical method was demonstrated to have reliability, reproducibility, and improved run-time and separation of xanthophylls. This method was used to confirm the localization of carotenoids in endosperm and to determine the effects of drying and storage on carotenoid levels in maize grain. A preliminary trial using room temperature drying indicated that while carotenoid profiles remain stable during storage, carotenoid levels decrease significantly from initial levels between 3 and 6 months of storage, but then remain stable for another year. A more rigorous trial using three drying and storage regimes (freeze-drying and storage at −80 °C; room temperature drying and storage; 90 °C drying and room temperature storage) indicated that extreme caution is needed to maintain carotenoid levels in maize during handling and storage, but in situations where freeze-drying is not possible, high heat drying is no more detrimental than low heat drying.

KEYWORDS: Maize (*Zea mays L.*); carotenoids; lutein; zeaxanthin; HPLC; drying; storage; postharvest

INTRODUCTION

The carotenoids lutein and zeaxanthin have been recognized as important factors in disease prevention and especially in maintaining eye health. Of the estimated >700 carotenoids occurring in nature, only lutein and zeaxanthin are selectively incorporated into the macula, and their loss from this tissue is associated with the onset of age-related macular degeneration (ARMD), a condition that affects the center of the field of vision and the ability to see fine details (*1*−*3*). Lutein and zeaxanthin are thought to function as antioxidants and as blue light filters, protecting ocular tissues from phototoxic damage (*4*,*5*). Approximately 25% of the population over 65 years of age exhibits early signs of the precursor disease age-related maculopathy (ARM) (*6*) and has a higher risk of developing ARMD (*7*). Medical intervention options are not available, resulting in ARMD being the leading cause of legal blindness in older adults (*7*). Epidemiological studies suggest that people with higher intakes of lutein and zeaxanthin are better protected against ARMD (*8*), and improving the lutein and zeaxanthin dietary content is theorized to be one of the best options currently available to delay the onset of ARMD (*9*).

Lutein and zeaxanthin are the most abundant carotenoids in maize (*Zea mays L.*) (*10*), and since maize is important both as a human staple and as animal feed, it is an ideal source of dietary carotenoids. In North America, field maize is an important animal feed and industrial feedstock; in 2008−2009, 51% of the maize used domestically in the USA was used for feed, 32% for ethanol production and 16% for other industrial and food uses (*11*). Applications of high carotenoid maize as animal feed may be used to improve animal health and marketability and, in the case of laying hens, to pass benefits to consumers in the form of high carotenoid foods. Lutein from eggs is more bioavailable to humans than either lutein supplements or spinach, a food traditionally considered to be a good source of carotenoids (*12*). Furthermore, supplementing lutein levels in the diets of laying hens can enhance egg lutein levels (*13*). Marigold petal supplementation has been the traditional source of lutein for laying hens (*14*); however, as carotenoid levels are improved in field maize, high carotenoid maize grain could increasingly fill this role in the diet of North American laying hens.

Historically, work on maize carotenoids has been limited to available material rather than germplasm developed for high carotenoid levels (e.g., the survey of North American inbred lines by Kurlich and Juvik (*15*)). Research on improving carotenoid content has been conducted by several groups. For example, Egesel et al. (*16*) were able to determine combining abilities for several Corn Belt dent inbred lines and concluded that selection for improved carotenoid content can yield improved varieties. More recently, Harjes et al. (*17*) demonstrated the power of targeting specific steps of the metabolic pathway in order to achieve the desired carotenoid profile, high β-carotene maize, and showed the potential for marker assisted selection to improve carotenoid content within breeding pools. None of these studies

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 Published on Web 07/01/2010

examined postharvest handling and storage of high carotenoid maize.

In the present work, carotenoid levels were determined in high carotenoid maize after exposure to different drying treatments and storage methods using an analysis method modified from Moros et al. (*18*).

MATERIALS AND METHODS

Maize. Yellow dent inbred maize lines A619, CG102, CG60 were used, as well as several unreleased lines from the University of Guelph high carotenoid (HiC) program: HiC-5, HiC-7, HiC-23, and HiC-26. These HiC lines arose from several breeding crosses involving the University of Guelph inbred lines CG102, CG33 and the sister-line hybrid CG60/CG62 and exotic accessions from Uruguay (UR), Chile (CH) and Argentina (AR) (Burt et al. unpublished data). For analyses presented here unless otherwise specified, plants were field grown in 2005 and 2006 using conventional production practices, ears were self-pollinated using controlled pollination methods, and ears were hand harvested and dried at room temperature under low light. After harvest and shelling, seed samples were stored in a long-term storage facility in the dark at 4 °C and 35% relative humidity.

Carotenoid Extraction. The carotenoid extraction procedure was slightly modified from Kurlich and Juvik (*15*). Three subsamples of 10 kernels were taken per cob and ground to a fine powder with a cyclone mill (model 3010-030, UDY Corp., Fort Collins, CO) fitted with a 0.5 mm mesh and stored in the dark at −20 °C for 1 to 5 days before extraction. From each subsample a 0.5 g sample was extracted for 6 min at 85 °C with 95% ethanol containing 1.25 g/L 2,6-di-*tert*-butyl-4-methylphenol (BHT). The extract, including the solids, was then saponified with 125 μL of potassium hydroxide (1 g/mL) at 85 °C for 10 min, chilled on ice and brought to 10 mL with cold distilled water. Liquid−liquid extraction was performed with four washes of 3 mL of hexane. The hexane fractions were pooled and dried by Speedvac (SC210A, Savant, Ramsey, MN) at room temperature. The dried extract was then reconstituted with three 100 μL washes of 2:1 methanol:methyl-*tert*-butyl ether (MTBE), which were pooled and filtered through a 0.2 μm PTFE filter into amber glass for HPLC analysis. Dry sample weights were determined by oven-drying the final extraction pellet to constant weight.

HPLC Analysis. The analysis method was modified from Moros et al. (*18*)) and performed using an Agilent 1100 system equipped with a 250 × 4.6 i.d. mm, 3 μm C30 carotenoid YMC column (Waters Corp, Milford, MA). The solvent system was composed of methanol (A) and MTBE (B). Starting conditions were 75% A, 25% B. The starting conditions were held for 3 min postinjection, followed by the gradient increasing linearly to 60% B in 18 min. The system was returned to the starting conditions in 5 min and allowed to re-equilibrate for 14 min, for a total run time of 40 min per injection. The flow rate was 1 mL/min, and detection occurred at 450 nm. Peak identification was determined by comparison of retention times and absorbance spectra with commercial chromatography grade standards (primary standards, >99% purity) obtained from ChromaDex (Santa Ana, CA).

Monitoring of visible light absorption at 450 nm provided optimal detection of the carotenoid compounds in all samples. Reference calibration curves for quantification were established for lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene, with ranges from 0.2−50 μg/mL for α-carotene to 2.5−150 μg/mL for zeaxanthin and lutein. The response was linear ($R^2 > 0.99$) for all calibrated compounds. Quantification of 13-*Z*-zeaxanthin was based on the lutein standard because of its matching λ_{max} (444 nm), equivalent absorbance at 450 nm, and identical molecular mass. All samples were quantified within linear response ranges; on the rare occasions that the linear range was exceeded, samples were diluted and reanalyzed.

Method Validation. Accuracy and precision of the HPLC methodology was assessed by examining the coefficients of variation (CV, standard deviation/mean × 100%) associated with intraday and interdays for a single extract. The test sample was analyzed every eighth injection for a total of four repetitions over 22 h on each of 3 days; the three test days were spaced one week apart. The limit of detection (LOD) and limit of quantification (LOQ), respectively defined as 3:1 and 10:1 peak-to-noise ratio, were determined for each reported compound by analysis of serially diluted samples, performed in triplicate.

Analytical results were validated at the Guelph Food Research Centre laboratories of Agriculture and Agri-Food Canada (henceforth referred to as the validation lab). Representative samples were selected to cover the linear range of the calibration curve as determined by the University of Guelph laboratory (henceforth referred to as the analysis lab). These samples were split into two aliquots that were stored at −20 °C. One aliquot was run in each laboratory within 48 h of one another. Calibration curves for lutein, zeaxanthin, β-carotene, and β-cryptoxanthin were created independently at the two laboratories using the same commercial standards.

Postharvest Handling. A rigorous analysis of handling effects was conducted in 2006. Three cobs from individual plants of six genotypes planted in a RCB design were collected from the field two weeks prior to regular harvest date to avoid the excessive weathering during dry-down once grain is fully mature. The grain was hand shelled from each ear and randomly split into three samples. One sample from each cob was frozen at −80 °C for 48 h and then lyophilized for 48 h and subsequently stored at −80 °C. A second sample was oven-dried at 90 °C for 8 h and subsequently stored at room temperature (25 °C) in the dark. The third sample was dried in an oven at ambient temperature with air blowing for 72 h and subsequently stored at room temperature in the dark. Once all samples were dried, three replicates from each drying regimen for each genotype were analyzed. Analyses were repeated with three more replicates after four months of storage under the conditions described.

Statistical Analysis. All statistical analysis was performed using SAS 9.2 (SAS Institute Inc., Cary, NC). Coefficients of variation of the analytical method were determined using PROC MEANS. Analytical results between the two laboratories were compared by simple linear regression using PROC GLM. Significance testing was done by ANOVA with multiple comparisons ($\alpha = 0.05$) using PROC GLM; only planned comparisons were used and no posthoc significance adjustment was made.

RESULTS AND DISCUSSION

Chromatographic Separation. The analysis method protocol from Moros et al. (*18*) was simplified to use unmixed solvents as the mobile phases in the gradient system to improve ease of use and consistency from run to run. Shortening the run time revealed the presence of a peak coeluting with lutein in the original separation; this peak was subsequently fully resolved and identified as 13-*Z*-zeaxanthin based on the absorbance spectrum and relative retention time provided by Updike and Schwartz (*19*). This identification was subsequently verified by mass spectrum analysis (data not presented). Extraction of carotenoids under increased temperature has been shown to be necessary for efficient extraction (*20*); however, the formation of *Z*- isomers of lutein and zeaxanthin from the all-*E* molecule is an artifact of the added heat and processing (*19*). Therefore, it is important to properly separate and quantify the major *Z*-isomers in the extracts for accurate characterization of the experimental materials. With the modified mobile phase and solvent gradient, all compounds extracted under high temperature from corn were readily separated and quantified in our laboratory (**Figure 1**). The improved separation and identification of xanthophyll isomers and increased speed and simplicity of this method make this a significant improvement over the previously published method from which it was adapted (*18*). Further comparisons made to other published methods (*21*, *22*) can be seen in the Supporting Information.

Method Validation. Accuracy and precision of the HPLC methodology was assessed by examining the CV associated with intraday and interdays for a single extract (**Table 1**). Both intra- and interday CVs were minimal for the analysis, indicating that the chromatographic separation used was highly repeatable both within and between days.

Analysis of serially diluted samples and subsequent peak-to-noise ratio calculation generated LOD and LOQ values for a subset of identified compounds (**Table 2**). LOD and LOQ values

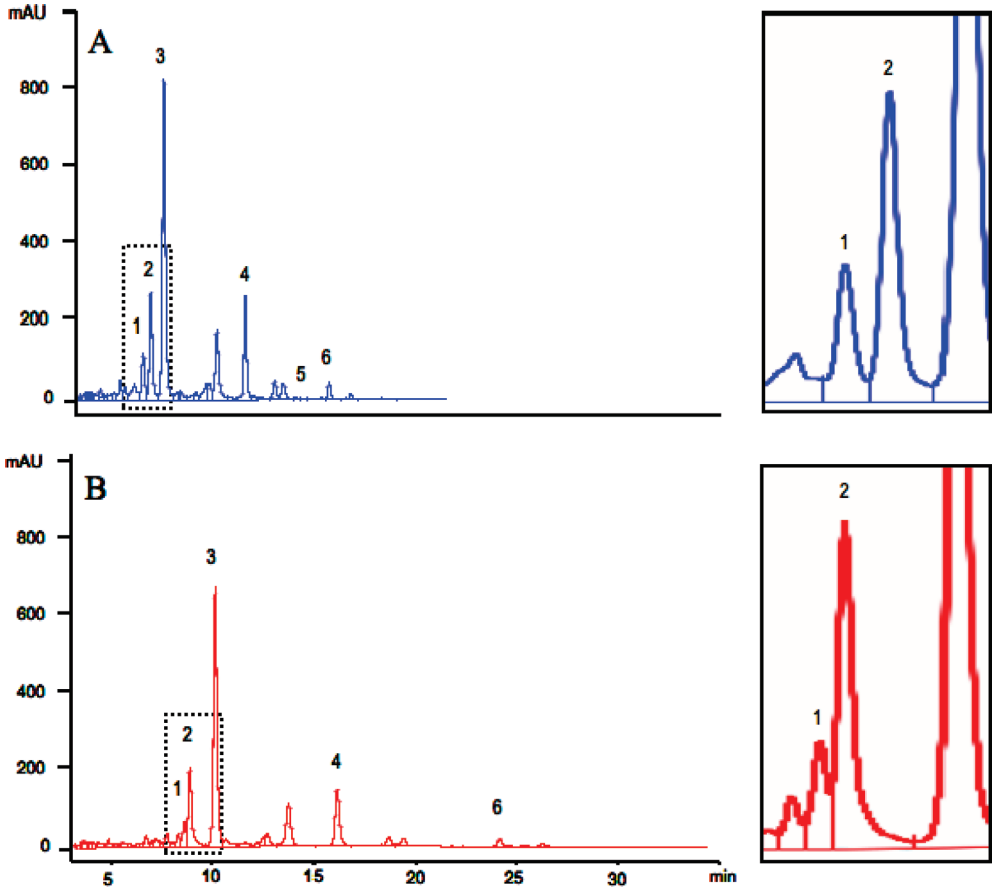

Figure 1. A comparison of chromatograms of HiC-21 (detection at 450 nm) using (**A**) the presented method, and (**B**) Moros et al. (*18*). Both methods use the same solvents (methanol, methyl-*tert*-butyl ether) and column. Enlarged section emphasizes the improvement in the separation of peaks 1 and 2 in the current method. Also, due to the narrowing, and therefore heightening, of peaks, peak 5 can be identified in **A**, but not in **B**; it is, however, too small to be quantified with either method. Key to peak identity: 1, lutein; 2, 13-*Z*-zeaxanthin; 3, zeaxanthin; 4, β-cryptoxanthin; 5, α-carotene; 6, β-carotene.

Table 1. Intraday and Interday Variation of HPLC Analysis, Determined by Performing Multiple Injections of the Same Extract

	variation (CV, SD/mean × 100%)			
	intraday			interday
	day 1	day 2	day 3	days 1−3
lutein	0.31%	0.27%	0.15%	0.08%
13-*Z*-zeaxanthin	0.79%	0.56%	0.27%	0.64%
zeaxanthin	0.35%	0.28%	0.16%	0.27%
β-cryptoxanthin	0.78%	0.41%	0.57%	0.66%
α-carotene	0.45%	0.53%	0.28%	0.46%
β-carotene	1.19%	0.67%	0.66%	1.15%

Table 2. Methodological Limits of Detection and Quantification for HPLC

	limit of detection (μg/mL ± SD)	limit of quantification (μg/mL ± SD)
lutein	0.19 ± 0.16	0.57 ± 0.30
13-*Z*-zeaxanthin	0.15 ± 0.16	0.44 ± 0.27
zeaxanthin	0.22 ± 0.13	0.65 ± 0.33
β-cryptoxanthin	0.05 ± 0.07	0.14 ± 0.09
α-carotene	0.03 ± 0.04	0.10 ± 0.09
β-carotene	0.05 ± 0.05	0.15 ± 0.03

are reported as the lowest detectable and quantifiable amounts of a compound present in a 1 mL sample (i.e., total content of the sample, regardless of extract concentration). The LOD values of the early eluting compounds (lutein, 13-*Z*-zeaxanthin, and zeaxanthin) ranged from 0.15 to 0.22 μg, approximately 3 to 4 times higher than those of the later eluting compounds; LOQ values vary accordingly. This difference between early and later eluting compounds is due to increased baseline noise at the early phase of the HPLC analysis that decreases later in the run.

The analysis of split samples at two laboratories under the same chromatographic conditions demonstrated that the analysis was repeatable, and precise (**Figure 2**). Concentrations of the major components, lutein and zeaxanthin, were only slightly underestimated (1.3%, and 9.2%, respectively) by the "analysis lab" as compared to the "validation lab", whereas the minor constituents, β-cryptoxanthin and β-carotene, were overestimated (3.4% and 27.3%, respectively). With an average bias of less than 10% for the major carotenoid components, the results reported in this paper are considered to be acceptably reliable, although it should be noted that the β-carotene concentrations reported here might be overestimates.

Localization of the Carotenoids in the Kernel. Carotenoid accumulation in maize is generally highest in the endosperm (*24*). This was verified with two HiC lines (**Figure 3**); the embryo tissue in these lines comprised between 10 and 15% of the grain weight and contributed very little to the carotenoid contents of the grain. However, the carotenoid profile of the embryo tissue was different from that in the endosperm and whole grain. The profiles of HiC-23 and HiC-7 in the endosperm tissue were strongly biased toward zeaxanthin and lutein, respectively, whereas the profiles of the embryo tissues of both lines had more balanced zeaxanthin:lutein ratios. This difference between tissues can be attributed to the tighter

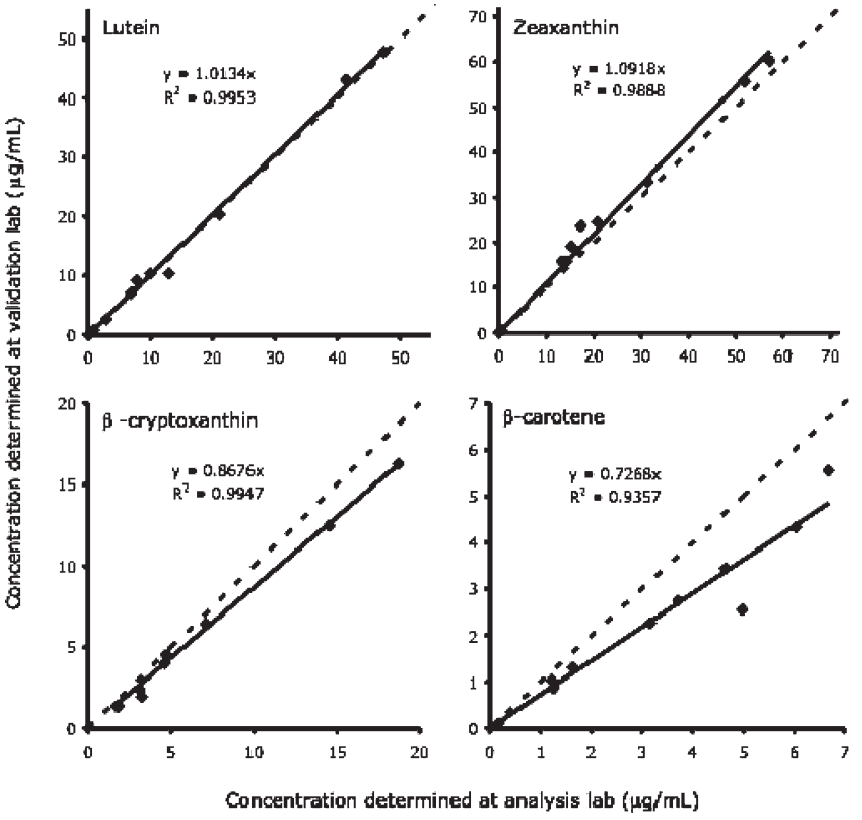

Figure 2. Validation of analytical protocol. Split samples were run at two different facilities within 48 h of each other. Dashed line represents a slope of 1 or perfect correspondence between the two laboratories. Solid line is the regression of the points; slope and r^2 value are shown.

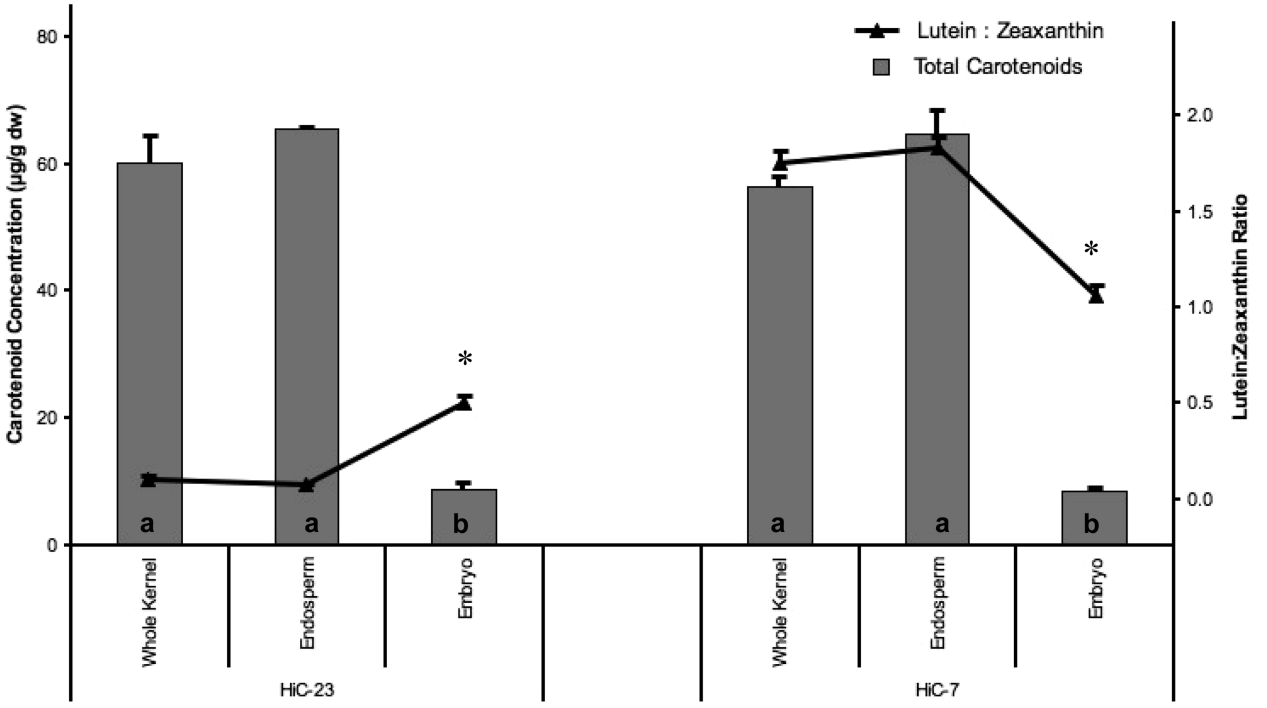

Figure 3. Total carotenoid concentrations and profiles (lutein to zeaxanthin ratio) by tissue type for two HiC inbred lines, HiC-7 and HiC-23. Each inbred line had an extreme profile type (high lutein and high zeaxanthin, respectively) in endosperm tissue. Profiles were significantly more balanced in the embryo of both inbred lines. Total bars labeled with different letters are significantly different. Ratio points labeled (*) are significantly different ($p < 0.05$) from the whole kernel profile. Data represent mean values ± SE ($n = 3$ biological replicates). Statistical comparisons are made only within inbred lines.

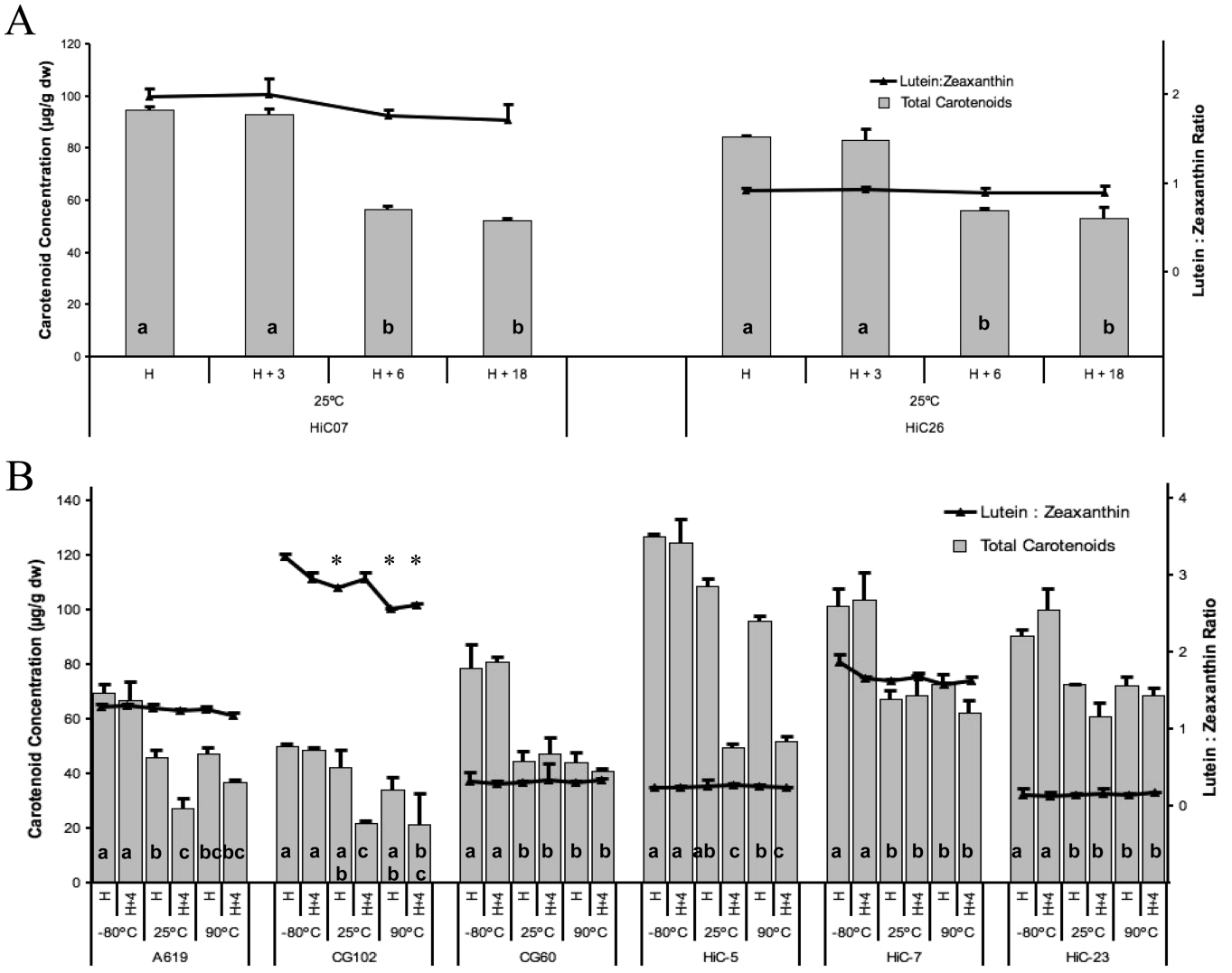

Figure 4. (**A**) Effects of storage time (3, 6, and 18 months) on carotenoid concentrations and profiles (lutein to zeaxanthin ratio). (**B**) Effects of drying treatments and storage (4 months) on carotenoid concentrations and profile (lutein to zeaxanthin ratio) for six maize genotypes. Total bars labeled with different letters are significantly different. Ratio points labeled (*) are significantly different ($p < 0.05$) from the initial time point for the −80 °C freeze-dried treatment. Statistical comparisons are made only within inbred lines.

regulation necessary in the embryo to ensure both photosynthetic function, which requires both lutein and zeaxanthin, and maintenance of appropriate seed dormancy and germination as regulated by the plant hormone abscisic acid (*23*), an eventual product of the zeaxanthin side of the carotenoid biosynthetic pathway.

Effects of Drying Temperature and Storage on Carotenoid Levels and Profiles. Drying and storage temperature are considered important in the stability of carotenoid compounds (*25*); however, this assumption has been only partially tested with maize grain. Quackenbush (*26*) demonstrated that the carotenoid levels of oven-dried (100 °C) maize grain decrease in room temperature (25 °C) and cold (7 °C) storage; drying temperature and alternative handling methods were not investigated. Here, an initial experiment to track degradation was performed using our standard handling methods: cobs were hand harvested and dried at room temperature under low light, followed by storage in a long-term facility in the dark at 4 °C and 35% relative humidity. Two genotypes, HiC-7 and HiC-26, were used since seed was available; these were sampled in triplicate after 0, 3, 6, and 18 months of storage. Under these conditions the total carotenoid concentration in both HiC-7 and HiC-26 genotypes remained stable between 0 and 3 months, but declined significantly by 6 months to a level that remained stable over the next year (**Figure 4A**). Quackenbush (*26*) likewise found that pigment losses were most rapid between 4 and 8 months of storage and declined during the later part of the storage period. While the total carotenoid concentrations decreased by approximately 40% in HiC-7 and 35% in HiC-26, the ratio of lutein to total zeaxanthins (i.e., the sum of zeaxanthin and 13-*Z*-zeaxanthin) did not change, indicating that the relative decline in the various carotenoids was similar.

In a more rigorous postharvest storage experiment (**Figure 4B**), we chose a high heat drying temperature (90 °C), a careful handling temperature (ambient temperature of 25 °C) and a gold standard (freeze-drying). The immediate effects of both 25 and 90 °C, compared to freeze-drying, were significant for all genotypes except for CG102 and HiC-5 (90 °C only). Surprisingly, none of the genotypes exhibited greater carotenoid loss at 90 °C than at 25 °C, indicating that extreme caution is needed to maintain carotenoid levels in maize samples, and that in situations where freeze-drying is not possible, high heat drying is not more detrimental than low heat/no heat drying.

The stability of total carotenoid concentrations under storage was also assessed with these samples. The freeze-dried material

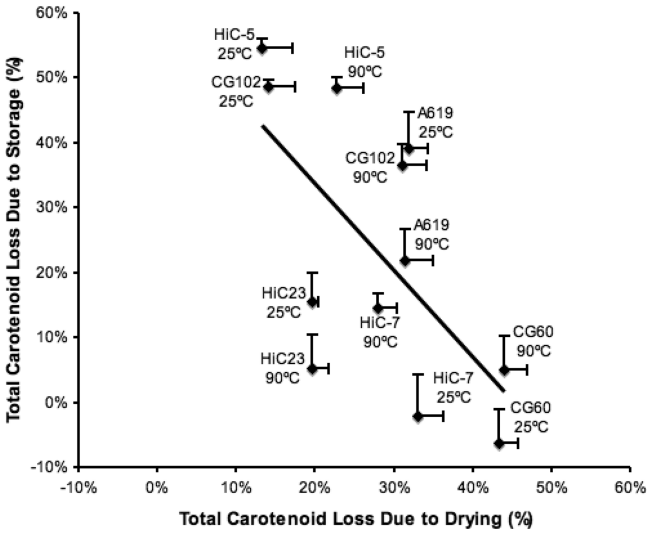

Figure 5. Comparison of carotenoid losses due to drying and storage. Regression line ($y = -1.332x + 0.603$) is shown, $r^2 = 0.401$. Each datum is labeled by genotype and drying temperature. Data represent mean values ± SE ($n = 3$ biological replicates). Data are replotted from **Figure 4B**.

was stored at −80 °C, and the other two drying treatments were stored at 25 °C, in the dark in all cases. At the final time point total carotenoid losses ranged from 24% for HiC-23 at 90 °C to 61% for HiC-5 at 25 °C; similarly, Weber (*27*) showed a mean carotenoid loss of 42 ± 4% in four inbred lines after six months of storage at room temperature. In the present study, in all cases the freeze-dried samples stored at −80 °C were not significantly decreased after four months of storage (**Figure 4B**). However, for the other drying treatments, there tended to be an inverse relationship between percent total carotenoid loss due to drying and storage for each sample, and the genotypes could be divided into three groups: those with high losses due to drying and low loss due to storage (CG60, HiC-7); those with low losses due to drying, but high loss due to storage (CG102, HiC-5); and intermediate genotypes with moderate losses due to both drying and storage (A619, HiC-23) (**Figures 4B** and **5**). These data suggest genetic differences in the kinetics of carotenoid loss during storage, but further research is required to assess whether these differences are maintained or disappear with additional storage.

Carotenoid profiles, as approximated by the ratio of lutein to zeaxanthins, were compared within each genotype across the drying and storage effects (**Figure 4B**). Compared to the initial measure of the freeze-drying treatment, all the genotypes, except CG102, did not exhibit any significant effect of drying treatment or storage time on the ratio. In CG102, the lutein to zeaxanthin ratio was significantly decreased by drying at 25 °C and by drying at 90 °C without or with storage at 25 °C for four months. It should be noted that CG102 is the genotype with the highest ratio of lutein to zeaxanthins of all the genotypes examined here, and that the use of the ratio exaggerates differences in a high lutein, low zeaxanthin profile. Statistical analysis of the inverse ratio (zeaxanthins to lutein) did not detect significant differences for any genotype across all treatments and time points; furthermore, differences in percent lutein or zeaxanthin were not observed. The ratio of lutein to zeaxanthins was presented here in preference to these other measures in order to provide as much information as possible about the overall carotenoid profile in a single measure. Therefore, it seems that there is little effect of drying or storage on the carotenoid profiles of most, if not all, maize lines.

High carotenoid maize has great potential as a source for carotenoid supplementation in animal feeds. In order for this to be realized, carotenoid contents must be improved through focused breeding efforts, accurate reliable and validated analysis methods must be utilized, and information on the impact of postharvest handling and storage on carotenoid concentration and composition must be provided to end users. Our findings suggest the potential to develop high carotenoid maize inbred lines that have good stability of carotenoids during drying and storage.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical support of Honghui Zhu who ran the validation samples at the Guelph Food Research Centre of Agriculture and Agri-Food Canada.

Supporting Information Available: Chromatograms of HiC-21. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review January 14, 2010. Revised manuscript received March 25, 2010. Accepted June 8, 2010. Financial support from the Natural Sciences and Engineering Research Council of Canada, Ontario Ministry of Agriculture, Food and Rural Affairs, Ontario Corn Producers' Association, Canadian Foundation for Innovation, and Ontario Innovative Trust is acknowledged.