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Evaluation of Analytical Methods for Carotenoid Extraction from Biofortified Maize (*Zea mays* sp.)

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Biofortification of maize with β-carotene has the potential to improve vitamin A status in vitamin A deficient populations where maize is a staple crop. Accurate assessment of provitamin A carotenoids in maize must be performed to direct breeding efforts. The objective was to evaluate carotenoid extraction methods and determine essential steps for use in countries growing biofortified maize. The most reproducible method based on coefficient of variation and extraction efficiency was a modification of Kurilich and Juvik (1999). Heat and saponification are required to release carotenoids from biofortified maize and remove oils interfering with chromatographic analysis. For maize samples with high oil content, additional base may be added to ensure complete saponification without compromising results. Degradation of internal standard before carotenoids were released from the maize matrix required the addition of internal standard after heating to prevent overestimation of carotenoids. This modified method works well for lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene.

KEYWORDS: **β-Carotene; carotenoids; corn; extraction; maize; xanthophylls**

INTRODUCTION

Maize is a staple crop that provides food to much of the world's population. In vitamin A deficient populations that consume predominantly plant-based diets, improving the concentration of provitamin A carotenoids, such as β-carotene, in staple food crops could potentially improve vitamin A status. Provitamin A carotenoids in typical maize include α-carotene, β-carotene, and β-cryptoxanthin; however, concentrations are typically low and range from 0 to 0.70, 0.07 to 1.46, and 0.07 to 1.05 μg/g and average 0.09, 0.31, and 0.41 μg/g, respectively (*1*). In addition to these carotenoids, maize also contains 0.4 to 32 μg/g lutein and 0.5 to 28 μg/g zeaxanthin, which are known antioxidants (*1*). Efforts by maize breeders have increased the provitamin A carotenoid content, e.g., 11 μg/g, and these carotenoids are nutritionally available (*2*). In order to direct breeding efforts of biofortified maize, the accurate assessment of carotenoids, especially β-carotene, is essential.

Compared to most carotenoid-containing foods, maize has high protein and oil content, i.e., 8−11 and 3−18% dry weight, respectively (*3*). Typical orange carrots have a high β-carotene concentration, i.e., 130 μg/g (*4*), and their composition is distinctly different from maize. On a fresh-weight basis, carrots have <1% protein and <0.5% fat (*5*), and the form of β-carotene is crystalline (*6*). Large differences between maize and carrot composition and matrix suggest that alternative extraction procedures are required.

Carotenoid analysis of foods is inherently difficult due to a large number of naturally occurring carotenoids, highly variable composition of foods, wide ranges of carotenoid concentrations, and isomerization and degradation of carotenoids prior to and during analysis (*7*, *8*). Errors associated with chromatography are minor (*9*), but errors from extraction procedures are potentially significant (*7*). With a variety of published methods available, it is difficult to select appropriate protocol for standardization between laboratories.

The most widely accepted methods involve extraction of carotenoids with one or more organic solvents including hexanes, tetrahydrofuran, methanol, ethanol, ethyl acetate, *n*-butyl-alcohol, and petroleum ether (*10*−*18*). Many procedures require freeze-dried material (*10*, *12*, *13*, *16*), saponification to remove lipids and chlorophylls (*11*, *12*, *15*, *18*), and the use of antioxidants, e.g., butylated hydroxytoluene (BHT) or pyrogallol (*11*, *16*, *18*). Less frequently used procedures utilize enzymes (*17*) and supercritical fluid extraction (*16*).

To continue investigations on the bioefficacy of provitamin A carotenoids in biofortified maize and standardization of protocol for developing countries, a thorough examination of carotenoid extraction procedures from maize was performed. The methods chosen were from HarvestPlus (*19*), Ben-Amotz and Fishler (*10*), Panfili et al. (*18*), Kurilich and Juvik (*21*), and one unpublished method. In addition to established procedures, some methods were modified to verify importance of specific steps. HarvestPlus is an international, multidisciplinary, research program that seeks to reduce micronutrient malnutrition through biofortification of staple crops. They have published a

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handbook of recommended carotenoid analysis procedures for staple crops including sweet potato, cassava, and maize using techniques available in developing countries (*19*). Their procedure, as well as the procedure by Ben-Amotz and Fishler, does not require saponification or heat, while the methods by Kurilich and Juvik and Panfili et al. do.

MATERIALS AND METHODS

Maize Samples. High β-carotene maize kernels were generously provided by Torbert Rocheford (University of Illinois at Urbana−Champaign). Upon receipt, maize was stored at −80 °C. Maize kernels were ground with a C&N laboratory hammer mill #8 (Christy-Norris, Ltd., Ipswich, U.K.) to pass a <1 mm sieve (particle size < 0.7 mm). Maize was further ground before or during extraction procedures using a mortar and pestle. Samples of maize were analyzed at least in triplicate by different methods to determine the optimum protocol for carotenoid analysis. All sample preparations, extractions, and analyses were performed under gold or UV-filtered white fluorescent lighting.

HarvestPlus Method (*19*). Dried ground maize (∼3 g) was hydrated at room temperature in water (∼10 mL) for 30 min followed by addition of acetone (∼20 mL) for 15 min. Carotenoids were extracted by grinding the mixture in a mortar and pestle with ∼50 mL acetone. The residue was vacuum filtered in a Buchner funnel equipped with filter paper (Whatman #2 filter paper, Middlesex, U.K.). The residue was returned to the mortar, and the procedure was repeated until the residue was nearly colorless, usually once more. One-third of the filtrate was transferred to a separatory funnel containing 20 mL of petroleum ether, to which 300 mL of distilled water was added. After the aqueous and organic layers separated, the aqueous layer was discarded. The procedure was repeated for the remaining filtrate. The organic phase was washed 3 times with 200 mL of distilled water and passed through anhydrous sodium sulfate (∼15 g) into a round-bottom flask. The sample was concentrated with a rotary evaporator and dried under argon.

Samples, extracted and prepared using the original procedure, were also analyzed on a Resolve C18 column (5 μm, 3.9 × 300 mm, Waters Corporation, Milford, MA) according to published procedures (*20*, *21*). To determine the effect of extraction solvent, the HarvestPlus method was also performed using hexanes and hexanes/ether mixture (25:75 v/v) instead of acetone.

Ben-Amotz and Fishler (*10*) Method. The maize sample (0.6 g) was extracted twice with tetrahydrofuran/methanol (5 mL, 50:50 v/v) by mixing and centrifugation. Hexanes (10 mL) and sodium chloride (2 mL, 10% w/v) were added to the combined organic layers. Rather than using a separatory funnel, layers were separated in a large glass test tube (50 mL). The organic layer was transferred to a new tube and dried under argon.

Kurilich and Juvik (*21*) Method. Carotenoids were released from dried maize (0.6 g) by adding ethanol (6 mL) containing 0.1% BHT (w/v), mixing by vortex for 20 s, and placing in an 85 °C water bath for 5 min. Potassium hydroxide (120 μL, 80% w/v) was added to the heated ethanol−maize mixture, to saponify potentially interfering oils. Samples were mixed by vortex and returned to the 85 °C water bath for 10 min with an additional mixing at 5 min. After saponification, samples were immediately placed in ice, and cold deionized water (∼3 mL) was added. Carotenoids were extracted 3 times with hexanes (∼3 mL) using centrifugation (1200*g*) to separate the layers. Combined organic layers were washed with deionized water (∼3 mL), and the organic layer was removed to a new test tube. The remaining aqueous layer was extracted twice more with hexanes. The combined organic layers were dried under argon.

Several modifications were performed to improve chromatography and verify importance of saponification and heating steps. Modification of the saponification process included addition of 500 μL of potassium hydroxide (80% w/v) instead of 120 μL. Modifications of the heating steps included changing the extraction and saponification temperature from 85 °C to 23 or 60 °C. All temperature changes were performed with the additional base modification described previously.

Panfili et al. (*18*) Method. The maize sample (2 g) was placed in a screw-capped vial. Ethanolic pyrogallol (5 mL, 60 g/L), ethanol (2 mL, 95% v/v), sodium chloride (2 mL, 10 g/L), and potassium hydroxide (2 mL, 600 g/L) were added. Samples were heated in a 70 °C water bath for 45 min with mixing every 5−10 min. Samples were transferred to an ice bath immediately following saponification. Sodium chloride (15 mL, 10 g/L) was added. The carotenoids were extracted twice with hexanes/ethyl acetate (15 mL, 9:1 v/v), and the combined organic layers were dried using rotary evaporation.

Unpublished Method. This unpublished method is very similar to the Kurilich and Juvik method, but it has a few major modifications. Carotenoids were released from dried ground maize (0.6 g) by heating at 50 °C for 10 min in ethanol with BHT (0.1% w/v). The carotenoids were extracted from the maize twice using petroleum ether/diethyl ether (6 mL, 4 mL, 2:1 v/v) and combined in a new test tube. The carotenoid extract was saponified with ethanolic potassium hydroxide (1 mL, 40% w/v) on ice for 2 min and at room temperature for 3 min. Next, distilled water (3 mL) was added, and the organic layer was removed to a new tube. The remaining aqueous layer was further extracted twice more with petroleum ether/diethyl ether (5 mL, 3 mL, 2:1 v/v), and the combined organic layers were dried under argon.

Internal Standard. β-Apo-8′-carotenal (∼100 to 250 μL, 6.5 mg/L) was used as an internal standard (IS) and chosen based on its carotenoid characteristics, retention time, and resistance to saponification. β-Apo-8′-carotenal was purchased from Sigma-Aldrich (St. Louis, MO) and purified as described for carotenoid standards below. In the HarvestPlus procedure, it was added prior to grinding in a mortar and pestle. In the Kurilich and Juvik method, it was added with the initial addition of ethanol (preheat) or immediately following heated saponification (postheat). In the Panfili et al. and Ben-Amotz and Fishler methods, it was added after the first addition of solvent, ethanolic pyrogallol and tetrahydrofuran/methanol, respectively.

Carotenoid Analysis Using Reverse-Phase HPLC. HPLC procedures for analysis of carotenoids in maize were adapted from published procedures (*22*, *23*). Samples from all procedures were reconstituted in methanol/dichloroethane (500 μL, 50:50 v/v) and injected (50 μL) into the HPLC. A Waters HPLC system (Waters Corporation, Milford, MA) consisting of a guard column, C30 YMC carotenoid column (4.6 × 250 mm, 3 μm, Waters Corporation), 1525 binary HPLC pump, 717 autosampler, and a 2996 photodiode array detector was used. Solvent A consisted of methanol/water (92:8 v/v) with 10 mM ammonium acetate. Solvent B consisted of 100% methyl *tert*-butyl ether. Gradient elution was performed at 2 mL/min with the following conditions: 29 min linear gradient from 83% to 59% A, 6 min linear gradient from 59% to 30% A, 1 min hold at 30% A, 4 min linear gradient from 30% to 83% A, and a 4 min hold at 83% A. β-Carotene eluted at approximately 25 min. Chromatograms were generated at 450 nm. Identification of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene were determined using standards and with verification of absorption spectrum.

Standards of zeaxanthin and β-carotene were purchased as nutritional supplements (GNC, Inc., Pittsburgh, PA). β-Cryptoxanthin was purchased from CaroteNature, GmbH (Lupsingen, Switzerland), and lutein was a gift from Kemin Industries (Des Moines, IA). α-Carotene was purified from carrot extracts as described previously (*20*, *24*). All carotenoids were purified on a Resolve C18 column (3.9 × 300 mm, 5 μm, Waters Corporation) using acetonitrile:methanol:dichloroethane (80:10:10 v/v) at 1 or 1.5 mL/min to ensure > 99% purity. The concentration of each standard was calculated from the $E^{1\%}_{1cm}$ [2592 for β-carotene, 2800 for α-carotene, 2550 for lutein, 2386 for β-cryptoxanthin, and 2348 for zeaxanthin] at their respective maximum wavelengths near 450 nm (*25*).

Statistical Analysis and Calculations. Values are means ± SD. Methods were compared using ANOVA at $\alpha < 0.05$. Differences between methods were determined using least significant differences (LSD) at $\alpha < 0.05$. Precision of methods was compared using the coefficient of variation (CV). Extraction efficiency was calculated by dividing the peak area of the IS in the sample by the peak area of the IS initially placed in the sample and multiplying by 100. Corrected concentrations of carotenoids in maize were calculated by dividing the ng carotenoid/g maize by the extraction efficiency.

Table 1. Summary of Extraction Procedures and Experimental Conditions Used To Quantify Carotenoids in Maize[a]

method	*n*	saponification	modifications[b]	°C	IS addition	extraction efficiency (%)[c]	CV (%)
HarvestPlus (*19*)	7	no	none	23	initially	74 ± 13	26
Ben-Amotz and Fishler (*10*)	3	no	none	23	initially	85 ± 1	1.5
Kurilich and Juvik (*21*)	3	yes	none	85	initially	58 ± 1	4.4
Kurilich and Juvik	8	yes	additional base	85	initially	67 ± 9	15.4
Kurilich and Juvik	3	yes	additional base	85	postheat	95 ± 1	2.3
Kurilich and Juvik	6	yes	additional base	60	initially	77 ± 7	19.5
Kurilich and Juvik	6	yes	additional base	60	postheat	91 ± 7	8.6
Kurilich and Juvik	3	yes	additional base	23	initially	88 ± 2	5.4
Panfili et al. (*18*)	3	yes	none	70	initially	48 ± 6	0.6
unpublished method	4	yes	none	50	postheat	91 ± 1	4.0

[a] Extraction efficiency is reported for each of the methods using β-apo-8′-carotenal as an internal standard (IS). Coefficient of variation (CV) of the corrected β-carotene concentration is reported to indicate the precision of the procedures. [b] Modifications to the saponification procedure involve additional potassium hydroxide (500 μL, 80% v/v). [c] Mean ± SD of *n* determinations. Percent extraction efficiency is calculated by dividing the peak area of the IS in the sample chromatogram by the peak area of the IS analyzed alone and expressed as a percent.

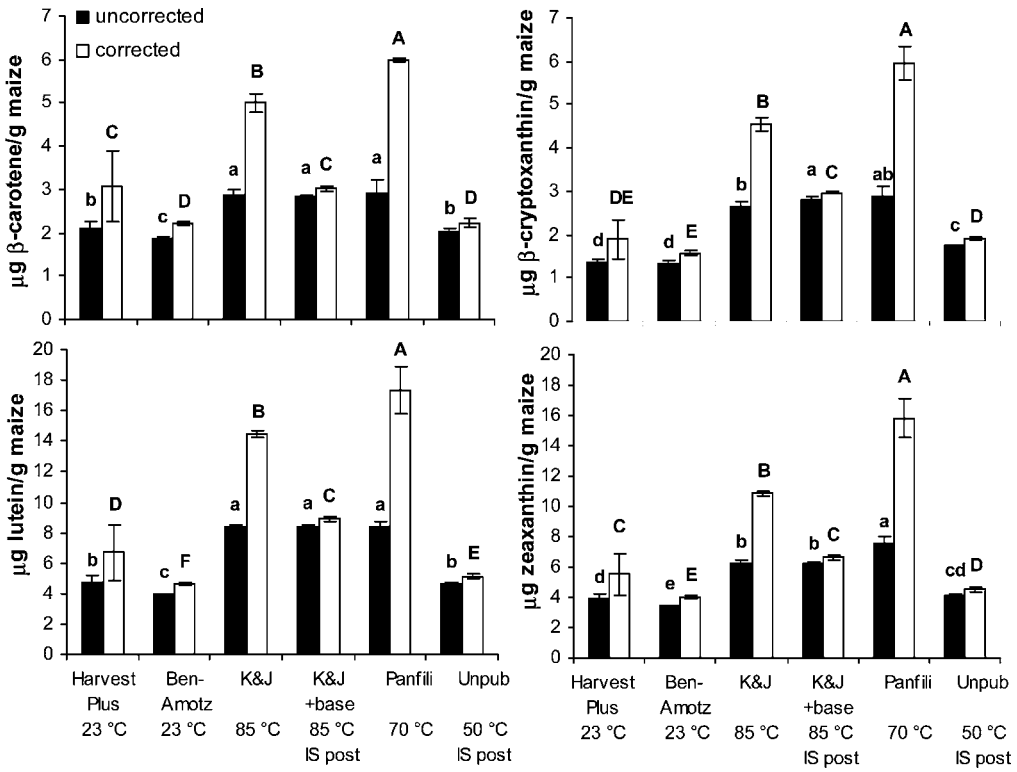

Figure 1. Uncorrected and corrected concentrations of β-carotene, β-cryptoxanthin, lutein, and zeaxanthin in high β-carotene maize using the HarvestPlus, Ben-Amotz and Fishler, Kurilich and Juvik (K&J), Kurilich and Juvik with additional base and internal standard (IS) added after heating and saponification steps, Panfili et al., and unpublished (unpub; IS added after heating and saponification steps) procedures. Correction of concentrations was made by dividing the uncorrected concentrations by their extraction efficiencies and multiplying by 100. Different capital letters represent different corrected carotenoid concentrations ($P < 0.05$). Different lowercase letters represent different uncorrected carotenoid concentrations ($P < 0.05$).

RESULTS AND DISCUSSION

The major carotenoids identified in the maize were the provitamin A carotenoids β-carotene and β-cryptoxanthin and the xanthophylls lutein and zeaxanthin. Uncorrected β-carotene and β-cryptoxanthin concentrations analyzed using selected methods (**Table 1**) ranged between 2 and 3 μg/g maize (**Figure 1**). Uncorrected lutein and zeaxanthin concentrations ranged from 3.4 to 8.4 μg/g maize (**Figure 1**). Minor amounts of α-carotene and 9- and 13-*cis* β-carotene were also identified. Total corrected provitamin A carotenoids in this maize were 6.3 μg/g. These carotenoids are excluded from the results due to chromatographic peaks near detection limits using some of the extraction procedures. Highly variable extraction efficiencies (**Table 1**) among procedures with similar uncorrected values resulted in highly inconsistent corrected values. This variability is primarily due to destruction of the IS during heat and saponification procedures. Typical yellow maize (2 μg provitamin A carotenoids/g maize) and a dark-orange maize with very high carotenoid concentrations (11 μg provitamin A carotenoids/g maize) were also analyzed by HarvestPlus, Kurilich and Juvik, and unpublished methods. These comparisons resulted in the same conclusions as those reported with high-β-carotene maize.

HarvestPlus. Although not readily apparent from the results, there were several analytical issues with the HarvestPlus method. The major concern was the presence of oil in the extract. When 3 g maize was used for analysis, approximately 120 μL of oil remained after the sample was dried. Upon reconstitution,

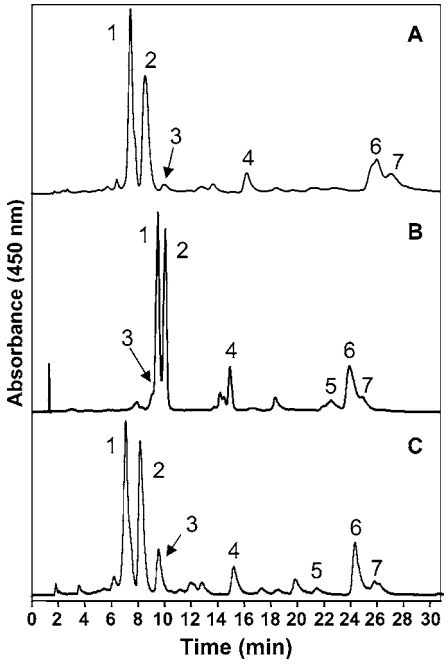

Figure 2. Chromatograms of carotenoids in biofortified orange maize extracted using the HarvestPlus method and analyzed using a C30 YMC column (**A**), extracted using the HarvestPlus method and analyzed using a Resolve C18 column (**B**), and extracted using the Kurilich and Juvik method with additional base and analyzed on a C30 YMC column (**C**). Peaks are (1) lutein, (*2*) zeaxanthin, (3) β-apo-8′-carotenal, (4) β-cryptoxanthin, (5) α-carotene, (6) all-*trans*-β-carotene, and (7) *cis*-β-carotene. Both columns are manufactured by Waters Corporation (Milford, MA).

samples appeared to be adequately dissolved, but partitioned into two phases when placed in the HPLC-autosampler kept at 4 °C. Apparent extraction efficiencies for these samples were as high as 157% due to variable partitioning of all carotenoids in the autosampler vial. This phenomenon resulted in huge quantification errors among the carotenoids due to differences in polarity of the IS and the carotenoid of interest. In order to prevent partitioning, the autosampler temperature was set at 16 °C and used for reported carotenoid concentrations (**Figure 1**). Even when the autosampler was set at 16 °C, calculated extraction efficiencies remained highly variable and ranged from 45 to 111%. Another issue, likely due to excess oil, was peak broadening in the chromatogram, which resulted in poor separation of *cis*- and *trans*-β-carotene (**Figure 2A**).

Several modifications were attempted with this method without success. When the extraction solvent was changed to hexanes or a hexanes/ether mixture (25:75 v/v), recovery rates decreased to ~25% (data not shown). Samples were also analyzed on a Resolve C18 column, but chromatographic resolution of all carotenoids on this column was poor under these conditions (**Figure 2B**). Subsequent injections resulted in deterioration of the column and decreasing retention times of β-carotene.

Although carotenoids in maize are generally not present in the ester form, saponification is a necessary step for maize when reverse-phase HPLC is used for analysis. Maize with lower oil content may present less of a problem. In addition, extraction using acetone, rather than hexanes or hexanes/ether, is preferable for this procedure. Addition of heat (85 °C) to the initial hydration step, an optional step suggested by HarvestPlus, also may improve values, but will decrease extraction efficiency if IS is added prior to heating. Due to complications with interfering oil in the biofortified maize, this was not attempted.

Ben-Amotz and Fishler. Uncorrected carotenoid concentrations from the Ben-Amotz and Fishler method were the lowest of any procedure. Extraction efficiencies were relatively high due to the lack of saponification or heat used in the method. Samples dried completely prior to HPLC analysis indicating that the oil fraction was not completely extracted from the sample. Although elimination of oil from samples improved analysis, β-carotene associated with the oil fraction was not extracted. Although unlikely, two potentially significant modifications (i.e., absence of freeze-drying and grinding with mortar and pestle without liquid nitrogen) were made to this procedure that could affect the concentration of β-carotene extracted. These modifications were made because freeze-drying and liquid nitrogen are not available in all laboratories. This procedure was originally designed and used on a wide variety of vegetables and fruits, including canned sweet corn. This method is probably adequate for foods with less difficult matrices, but performed here it underestimates carotenoids in maize.

Kurilich and Juvik. The Kurilich and Juvik method resulted in greater uncorrected β-carotene concentrations compared with the HarvestPlus, Ben-Amotz and Fishler, and unpublished methods ($P < 0.05$, **Figure 1**). With the high β-carotene maize reported in **Figure 1**, the oil appeared to be completely saponified, but when a dark-orange maize variety with higher oil and β-carotene content was used, saponification was incomplete (data not shown). The procedure was repeated with additional base (500 μL potassium hydroxide, 80% w/v). The uncorrected carotenoid concentrations did not differ, except for a slight increase in β-cryptoxanthin with the additional base, suggesting a slight positive effect on carotenoid extraction (**Figure 1**). Extraction efficiencies (IS added preheat) were greater with additional base, i.e., 67 ± 9% vs 58 ± 1% ($P < 0.05$), and oil was completely removed from the sample.

The Kurilich and Juvik procedure was repeated at 60 and 23 °C to determine the effect of heat on carotenoid release (**Figure 3**). Comparing the procedures at 85 and 23 °C showed a 30% increase in extraction efficiency and a 32% decrease in carotenoid extraction indicating heat is required for carotenoid release. β-Carotene extraction did not differ at 85 and 60 °C, but there was a significant increase in lutein, zeaxanthin, and β-cryptoxanthin at 85 °C ($P < 0.05$). Extraction efficiencies were significantly different at each temperature and increased with decreasing temperature, i.e., 67 ± 9, 77 ± 7, and 88 ± 2% at 85, 60, and 23 °C, respectively. Therefore, destruction of carotenoids in maize did not follow the same pattern as destruction of IS added preheat. This signifies that correction of carotenoid concentrations using an IS exposed to heat is invalid. The maize matrix likely has a protective effect on carotenoids during the heat and saponification steps, but without knowing the carotenoid concentrations in the maize before extraction, it is impossible to assess the degree of protection the maize matrix provides. Addition of the IS after heating allows correction for losses during transfer and other sample handling procedures and is assumed to be the more accurate method.

Panfili et al. Uncorrected carotenoid concentrations from the Panfili et al. method were similar to the Kurilich and Juvik method, except for zeaxanthin, which was greater than all other methods. Extraction efficiencies were the lowest of any procedure (48%), likely due to the long, 45 min, and hot, 70 °C, saponification step. The consistency in uncorrected carotenoid concentrations and inconsistency in extraction efficiencies between the Kurilich and Juvik and Panfili et al. methods further indicate that destruction of the IS does not mimic destruction

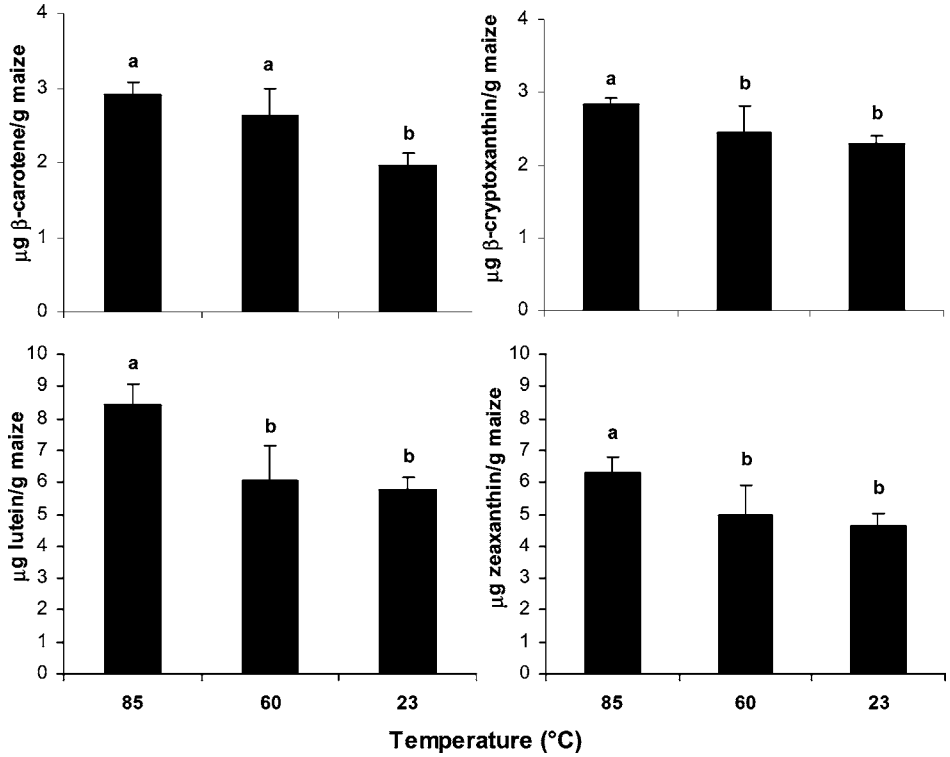

Figure 3. Uncorrected concentrations of β-carotene, β-cryptoxanthin, lutein, and zeaxanthin in high β-carotene maize using the Kurilich and Juvik procedure performed at 85, 60, and 23 °C. Different letters represent different uncorrected carotenoid concentrations ($P < 0.05$).

of carotenoids. For this method, corrected carotenoid concentrations are very high and most likely not accurate. Addition of the IS after heat and saponfication steps would likely improve extraction efficiencies, but due to the long saponification time of the Panfili et al. method, the Kurilich and Juvik procedure was the preferred method.

Unpublished Method. An unpublished method, similar to the Kurilich and Juvik method, was also performed. Instead of saponifying the sample prior to extracting the carotenoids from the maize, the carotenoids were extracted with petroleum ether/diethyl ether (2:1 v/v) before saponification and extracted again with the same solvents after saponification. Using this procedure, carotenoid concentrations were significantly lower than the Kurilich and Juvik procedure. It is hypothesized that the order, time, and temperature of saponification were the causes of lowered carotenoid concentrations.

Conclusion. Of the methods examined, the Kurilich and Juvik method at 85 °C (IS postheat) was the most reliable method to determine carotenoid concentrations in maize based upon higher extracted carotenoids, good extraction efficiencies, and a low CV. For maize samples, heat and saponification are required for complete extraction and improved HPLC analysis (**Figure 2C**). Saponification before removal of carotenoids from the maize matrix may also be important as lower carotenoid concentrations were obtained when saponification occurred after extraction. For maize samples with high oil content, additional base may be added to ensure complete saponification. Correction of carotenoid concentrations using extraction efficiency should be made by addition of IS after heating to prevent overestimation of carotenoids. This extraction method works well for lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene.

ABBREVIATIONS USED

BHT, butylated hydroxytoluene; IS, internal standard.

SAFETY

Use of a fume hood for volatile organic solvents is recommended.

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