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Analysis of Xanthophylls in Corn by HPLC

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An HPLC method was developed using the C-30 carotenoid column to separate and identify the major xanthophylls in corn (lutein, zeaxanthin, and β-cryptoxanthin). A photodiode array detector and a mobile phase consisting of methyl *tert*-butyl ether/methanol/water was used. All three xanthophylls eluted in less than 25 min. Yellow dent corn had a total xanthophyll content of 21.97 μg/g with lutein content of 15.7 μg/g, zeaxanthin content of 5.7 μg/g, and β-cryptoxanthin of 0.57 μg/g. Commercial corn gluten meal had a 7 times higher concentration of xanthophylls (145 μg/g), and deoiled corn contained 18 μg/g, indicating that the xanthophylls are probably bound to the zein fraction of corn proteins.

KEYWORDS: Xanthophylls; lutein; zeaxanthin; corn; HPLC

INTRODUCTION

Xanthophylls are pigments of the carotenoid family. Examples of xanthophylls are lutein, zeaxanthin, and β-cryptoxanthin, which are present in plants such as corn, marigolds, alfalfa, kale, and spinach, and astaxanthin which is found in fish, birds, and sea crustaceans. High concentration of xanthophylls are also present in egg yolks and the retina of the eye (*1*). In corn, xanthophylls are mostly found in the horny endosperm. The total xanthophyll content in whole corn is 11−30 mg/kg (*2*).

Whereas carotenes are hydrocarbons, xanthophylls are oxygenated hydrocarbon derivatives that contain hydroxy, keto, epoxy, methoxy, or carboxylic acid groups. The characteristic feature of the xanthophyll structure is the alternating double and single bonds that form the central part of the molecule (the chromophore). This constitutes a conjugated system in which the π-electrons are effectively delocalized over the entire length of the polyene chain (*3*). It is this feature that gives xanthophylls their shape, chemical reactivity, and light absorbing properties which result in various shades of red, yellow, and orange colors. This unique structure in turn allows the xanthophylls to act as both natural food colorants and as antioxidants. Several desirable health-related properties of xanthophylls have been identified, e.g., some show antitumor promoting activity, and suppression of tumor growth occurred in mice who were fed certain concentrations of lutein (*4*,*5*). Other studies have linked lutein and zeaxanthin to the prevention of age-related macular degeneration (AMD), a human disorder similar to cataracts that causes early blindness (*6*).

Several methods of analyzing xanthophylls in corn have been devised over the years. Quackenbush (*7*) analyzed the composition of xanthophylls in corn by separating them on a magnesia column and collecting several fractions of xanthophylls. Several xanthophylls were identified, including xanthophyll esters and other pigment fractions. A common method is the official AOAC method for xanthophylls and carotenes (43.018−43.023) using hot extraction and saponification (*8*). However, these methods measure total xanthophyll content because the individual xanthophylls and carotenes are not separated or identified.

Livingston (*9*) proposed a method of extraction conducted at ambient temperature followed by saponification with hexane/acetone solvent. The sample would then be analyzed by the same AOAC method previously described. This method resulted in higher values than the standard AOAC method.

Weber (*10*) developed an HPLC method for xanthophylls in corn. Samples were ground and saponified with KOH in ethanol. A hexane/toluene mixture was used to extract the xanthophylls. The column was a hand-packed 5-μm Ultrasphere silica column with an Adsorbosphere silica cartridge guard. Absorption was measured at 445 nm by a UV−vis spectrophotometer. This normal-phase HPLC system separated individual xanthophylls but coeluted the carotenes as one peak.

Kurilich and Juvik (*11*) modified Weber's method and used two conventional C_{18} reverse-phase columns to separate the carotenoids: a polymeric C_{18} reverse-phase column (Alltech OD52) was followed by a monomeric C_{18} reverse-phase column (Vydac 201TP54). These columns did separate the carotenoids but lutein and zeaxanthin were poorly resolved.

This paper reports on an HPLC method of analysis using a YMC-C30 column with a photodiode array detector (DAD). A similar system was used in our laboratory (*12*) with methyl *tert*-butyl ether/methanol/water as the mobile phase to separate carotenoids, tocopherols, and tocotrienols in red palm oil. Thirteen carotenoids were detected and quantified within 45 min. Thus, it should be possible to obtain good resolution of both polar xanthophylls and nonpolar carotenes with this system. Our focus was the analysis of lutein, zeaxanthin, and β-cryptoxanthin in various corn samples, including regular yellow dent corn, white corn, inbred corn, commercial corn gluten meal, and milled corn from a dry-grind ethanol producer.

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MATERIALS AND METHODS

Samples and Chemicals. Raw whole corn (yellow dent #2) was obtained from Anderson Grain Co., Champaign, IL. It assayed 14.9% moisture, 4.1% oil, and 7.6% protein (N × 6.25). The whole corn was ground in a hammer mill (Mikropul Mikro-pulverizer, Type SH) fitted with a 0.20-mm screen. About 70% of the particles were 250-μm size as determined with an RO-tap shaker and U.S. standard sieves. Corn gluten meal was obtained from A. E. Staley Mfg. Co., Decatur, IL. Ground whole corn (referred to as "dry-grind corn" in this paper) was obtained from a dry-grind ethanol plant (Nebraska Energy Co., Aurora, NE). Over 70% of the particles in dry-grind corn were 200-μm to 800-μm size. White corn was obtained from a farm in central Illinois through the courtesy of Terry Wolfe. Inbred high-zeaxanthin corn A632 was obtained from the Department of Crop Sciences at the University of Illinois through the courtesy of J. C.Wong. White corn and inbred corn samples were ground with a mortar and pestle.

Xanthophyll standards (lutein, zeaxanthin, and β-cryptoxanthin) were obtained from Extrasynthase Company, Genay, France.

The extractant used for sample preparation was 100% ethanol (EtOH) containing 0.1% (w/v) butylated hydroxy toluene (BHT, Sigma Chemical Co., St. Louis, MO) as an antioxidant. This solution was made fresh for every experiment.

Sample Preparation. Sample preparation was a modification of the procedure of Wong et al. (*13*). It was done under dim yellow lighting to protect the xanthophyll from light-induced degradation. For solid samples (e.g., corn), 600 mg of cold ground sample was placed in a 20 × 150 mm test tube and 6 mL of 0.1% BHT−EtOH solution was added to each test tube. For liquid samples, 3 mL of extract was placed in a 20 × 100 mm test tube and 6 mL of the BHT−EtOH solution was added. The test tubes were sealed with screw caps and placed in an 85 °C water bath for 5 min or until ethanol was brought to its boiling point. The test tubes were then removed from the water bath and 120 μL (60 μL for liquid samples) of 80% KOH was added to each tube. Samples were then vortexed and returned to the water bath for 10 minutes for saponification to occur.

After the samples were saponified, the test tubes were immediately placed in an ice bath to cool, and then 3 mL (or 1.5 mL for liquid samples) of cold deionized water was put into each test tube, followed by 3 mL of hexane (or 1.5 mL for liquid samples). The test tubes were then vortexed and immediately centrifuged at 2500 rpm (660*g*) for 10 min. The top layer was removed with a Pasteur pipet and added to a separate test tube. The hexane wash was repeated two more times. All hexane extracts were combined in the same test tube. The hexane was evaporated in a stream of nitrogen passed into the test tube until dry (typically 2 h). The residue was then solubilized in 200 μL of mobile phase A (100 μL for liquid samples). Samples were stored at −20 °C under nitrogen until injected into the HPLC column.

HPLC. The HPLC system was a Hewlett-Packard HPLC 3D System Series model 1050 A with a photodiode array detector, a Quanton pump system, and a HP 3D Chemstation program to record data. The column was a 4.6 mm × 250 mm C-30 Carotenoid column (YMC/Waters Inc., Wilmington, NC). A guard column (4 mm × 23 mm) containing the same packing material as the C-30 column was installed ahead of the carotenoid column. The solvents were HPLC grade methanol (Sigma) and methyl-*tert*-butyl-ether (MTBE, Fisher Scientific, Pittsburgh, PA). A gradient system was used involving two separately mixed mobile phases. Mobile phase A was methanol/MTBE/water (81:15:4) and mobile phase B was methanol/MTBE (9:91). The initial values were 100% of A and 0% B, to 50% A and 50% B in 45 min, followed by 100% B within 15 min. The flow rate was 1.0 mL/min during the entire run. All samples were injected via a 20-μL loop using a 100-μL syringe.

On the basis of the absorbance maxima for the xanthophylls shown in **Table 1**, detection was done at 450 nm and 445 nm by a model 1050A Hewlett Packard UV−vis photodiode array detector. Standard curves of lutein, zeaxanthin, and β-cryptoxanthin were constructed by plotting HPLC peak absorbance area vs concentration of the xanthophyll in the injected sample. Concentrations of the xanthophylls were measured against an absorption curve that had been determined independently as follows: the xanthophyll standards were individually dissolved in mobile phase A to the required concentration. The solvent was then evaporated under nitrogen to dryness. The residue was reconstituted in a known volume of 100% ethanol, usually 5 mL. The absorbance of this sample was measured in a Hewlett-Packard model 8543 UV−vis spectrophotometer. Extinction coefficient values of lutein, zeaxanthin, and β-cryptoxanthin in ethanol obtained from Wong et al. (*13*) are shown in **Table 1**. They were used to calculate the concentrations of the xanthophylls using Beer's Law.

Table 1. Extinction Coefficients (ϵ) and Maximum Wavelengths (λ_{max}) of Lutein, Zeaxanthin, and β-Cryptoxanthin in 100% Ethanol (*13*) and Calibration Equations for the C-30 Column

xanthophyll	λ_{max} (nm)	ϵ (dL/g·cm)	standard curve[a]	R^2
lutein	445	2765	$y = 71.034x$	0.9901
zeaxanthin	452	2416	$y = 130.79x$	0.9958
β-cryptoxanthin	452	2486	$y = 132.07x$	0.9734

[a] y = Area of peak (mAU); x = concentration (μg/mL).

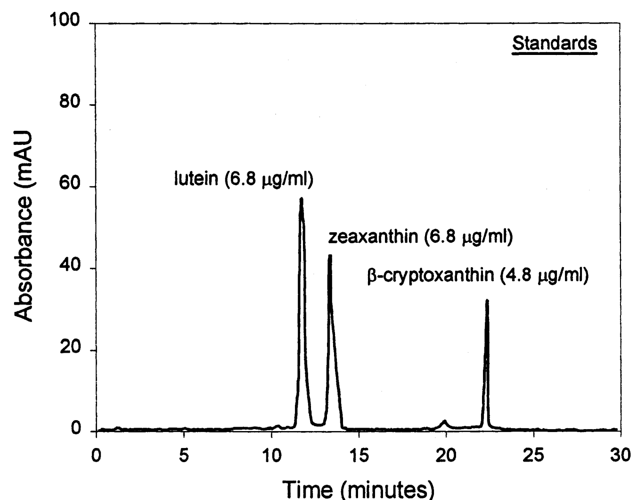

Figure 1. Elution profile of xanthophyll standards on C-30 column. Detection was done at 450 nm.

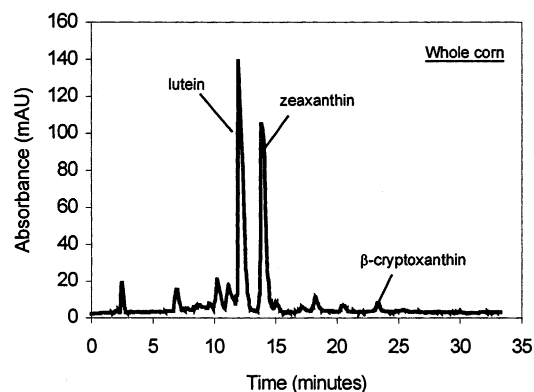

Figure 2. HPLC of whole corn on C-30 column. Detection at 450 nm.

RESULTS AND DISCUSSION

The elution profile of the xanthophyll standards with the YMC-C30 carotenoid column and reverse-phase chromatography is shown in **Figure 1**. The elution time was less than 25 min for lutein, zeaxanthin, and β-cryptoxanthin. With this HPLC column, the more polar xanthophylls, such as the dihydroxylcarotenoids lutein and zeaxanthin, elute before the monohydroxylcarotenoid β-cryptoxanthin. In contrast, the order of elution is reversed with the normal phase C-18 silica columns. Baseline separation was obtained for the xanthophylls. Sander et al. (*14*) reported that the first carotenoid eluted using the C-30 column and a similar mobile phase was the polar carotene

Table 2. Xanthophyll Content of Whole Corn, Corn Residue, and Deoiled Corn

sample	xanthophyll	replication	concentration (μg/g corn)	total xanthophylls (μg/g corn)	average (μg/g corn)
whole corn	lutein	1	14.68		
	zeaxanthin		5.38	20.47	
	β-cryptoxanthin		0.41		
	lutein	2	14.26		20.09 ± 0.38
	zeaxanthin		5.09	19.71	
	β-cryptoxanthin		0.36		
whole corn residue	lutein	1	1.28		
	zeaxanthin		0.46	1.92	
	β-cryptoxanthin		0.18		
	lutein	2	1.25		1.88 + 0.04
	zeaxanthin		0.44	1.88	
	β-cryptoxanthin		0.15		
deoiled corn	lutein	1	13.87		
	zeaxanthin		4.74	18.84	
	β-cryptoxanthin		0.24		
	lutein	2	12.75		17.96 ± 0.88
	zeaxanthin		4.09	17.08	
	β-cryptoxanthin		0.24		

Table 3. Xanthophyll Content (μg/g) of Corn Products, Inbred Corn, and White Corn

sample	lutein	zeaxanthin	β-cryptoxanthin	total xanthophylls
dry-grind corn	12.84 ± 0.84	2.97 ± 0.30	0.30 ± 0.02	16.11 ± 1.16
corn gluten meal	106.90 ± 1.41	34.26 ± 0.56	4.75 ± 0.09	145.91 ± 2.06
inbred corn A632	5.49 ± 1.12	6.83 ± 1.78	0.36 ± 0.16	12.68 ± 3.06
white corn	0.08	0.04	0	0.12

(astaxanthin) with a retention time of 11 min. **Table 1** shows the linear regression equations derived for the standard curves.

Identification of Corn Xanthophylls. Figure 2 shows the chromatogram of the whole corn after one BHT−EtOH extraction and three hexane washes as described in Materials and Methods. The peaks are well separated by the C-30 column. Identification was based on the order of elution, retention time, and spectra of absorbance maxima of a particular peak. These data were compared with information in the literature. The peaks identified in this analysis are shown in **Figure 2**.

Lutein was identified at a retention time of 12 min with absorbance maxima at 445 nm. This peak was confirmed by comparison with the peak of a lutein standard. The other polar xanthophylls identified were zeaxanthin and β-cryptoxanthin at retention times of 14 and 23 min, respectively. These peaks were confirmed by comparison with the pure compounds. The identities of the other peaks are unknown, even though we had previously identified as many as 13 carotenoids and 5 tocopherols with this column (*12*).

Individual values and averages are shown in **Table 2**. Good repeatability was observed with this method. The total xanthophylls (the sum of lutein, zeaxanthin, and β-cryptoxanthin) was 20.09 μg/g corn (**Table 2**). However, all the xanthophylls were not extracted from whole corn by using just one extraction step because the residual corn solids were slightly yellow in color. The residual corn solids were re-extracted and the analysis was repeated. An additional 1.88 μg xanthophylls/g corn was extracted, for a total of 21.97 μg xanthophylls/g corn. When the extraction was repeated a total of five times with the residual corn solids, the xanthophylls totaled 22.81 μg/g corn. Perhaps some xanthophylls were bound to corn components such as zein or trapped in the corn solids. In contrast, Weber (*13*) used a normal-phase C-18 column and obtained a total of 15.8 μg/g corn for both carotenoids and xanthophylls.

Processed Corn Products. The HPLC profile of commercial dry-grind corn is similar to that of whole corn shown in **Figure 2** because they were of the same yellow dent variety. The xanthophyll levels in dry-grind corn (**Table 3**) were lower, perhaps due to degradation of xanthophylls during storage, as the dry-grind corn samples had been stored for several months before analysis. Xanthophylls are sensitive to direct light, heat, oxygen, and low pH (*10*).

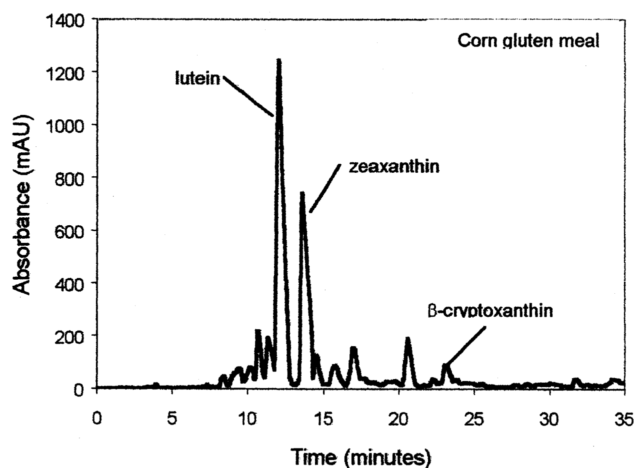

Figure 3. HPLC of commercial corn gluten meal on C-30 column. Detection was done at 450 nm.

Corn gluten meal (**Figure 3**) had the highest concentrations of lutein, zeaxanthin, and β-cryptoxanthin among all the samples we analyzed for this research. Total xanthophyll concentration was 145.91 ± 2.06 μg/g corn gluten meal (**Table 3**), about 7.2 times higher than whole corn assayed under similar conditions. This is not surprising considering that the protein content of corn gluten meal is about 60% (dry basis) compared to 7.6% protein in whole corn, about 7.9 times higher. This suggests that the xanthophylls are probably bound to a protein, probably zein which is ethanol soluble and has unusual structural and

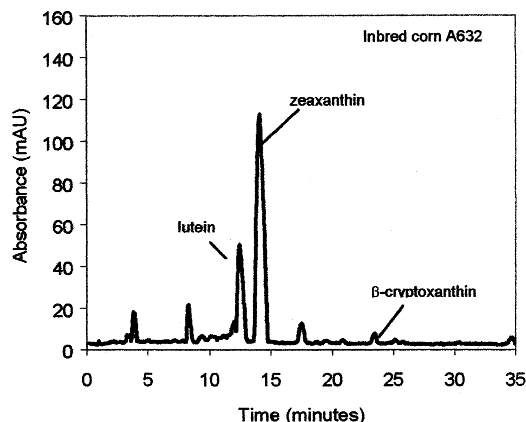

Figure 4. HPLC of inbred corn A632 on C-30 column. Detection at 450 nm.

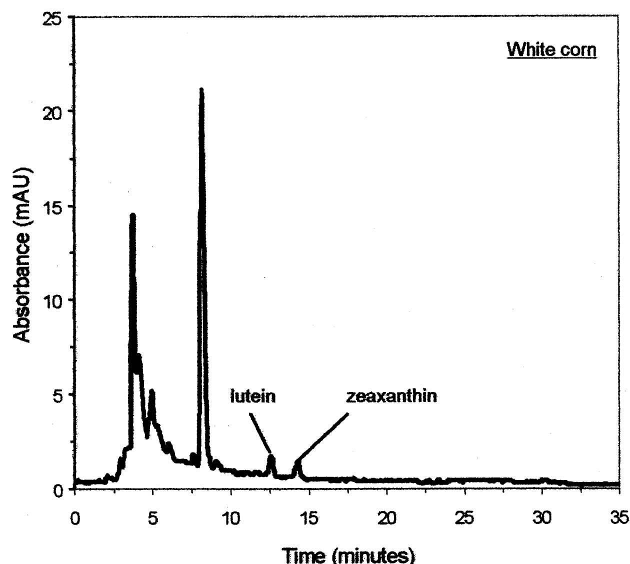

Figure 5. HPLC of white corn on C-30 column. Detection at 450 nm.

physical properties (*15*). Thus, during the corn wet milling process, the xanthophylls will go with the zein protein fraction which ends up in the corn gluten meal.

Deoiled Corn. The zein–xanthophylls interaction was confirmed by analyzing the deoiled meal after the oil had been extracted from corn by the Soxhlet procedure with *n*-hexane. The oil had a yellow color indicating that some of the xanthophylls were in the oil. The deoiled corn residue (i.e., the meal left after Soxhlet extraction containing 0.1% residual oil) had a total xanthophyll content of 17.96 ± 0.88 μg/gram deoiled corn (**Table 2**), compared to the original whole corn content of 21.97 ± 0.42 μg/gram corn. Thus, about 85% of the xanthophylls remained in the corn after the oil was removed. This is a further indication of possible strong hydrophobic interactions between the xanthophylls and zein.

Inbred and White Corn. Figure 4 shows the chromatogram of inbred corn A632. The main difference between this variety and regular corn (**Figure 2**) was that the zeaxanthin peak was larger than the lutein peak with the inbred corn. The total xanthophyll content was lower in inbred corn A632 (**Table 3**), possibly due to degradation because this sample had been stored under ambient conditions for more than one year prior to analysis.

HPLC analysis of white corn (**Figure 5**) showed small amounts of lutein and zeaxanthin for a total of about only 0.12 μg/mg (**Table 3**), which is 0.5% of the total xanthophyll content found in regular yellow dent corn. No other xanthophyll peaks (including no β-cryptoxanthin) were detected in the white corn at the 450-nm HPLC scan. The prominent peak with an elution time of 8 min is unknown.

In conclusion, HPLC using the C-30 carotenoid column is a good method of analyzing xanthophylls. To obtain satisfactory separation of xanthophylls, they must be completely extracted and released from their ester form. This is done by saponification which also eliminates contaminating substances such as lipids and proteins that could potentially plug the carotenoid column. The xanthophylls can then be collected in the hexane layers and the unknown concentrations determined from the standard curves. Because the xanthophylls are sensitive to direct light, heat, oxygen, and low pH (*10*), they must be protected during the analysis.

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