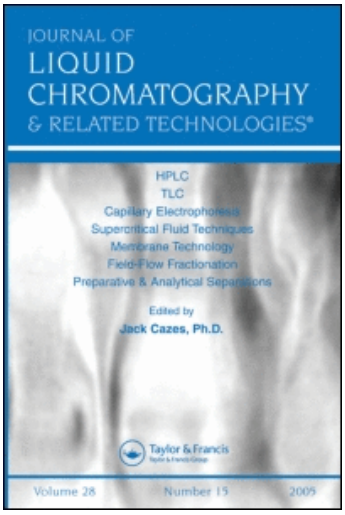

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Kale, Aniket V. and Cheryan, Munir(2007) 'Rapid Analysis of Xanthophylls in Ethanol Extracts of Corn by HPLC', Journal of Liquid Chromatography & Related Technologies, 30: 8, 1093 — 1104
To link to this Article: DOI: 10.1080/10826070601128469
URL: http://dx.doi.org/10.1080/10826070601128469

Journal of Liquid Chromatography & Related Technologies®, 30: 1093–1104, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070601128469

Rapid Analysis of Xanthophylls in Ethanol Extracts of Corn by HPLC

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Abstract: A rapid HPLC method for the identification of xanthophylls in ethanol extracts of corn was developed. This method requires only the extraction of xanthophylls using aqueous ethanol followed by injection of the microfiltered extract onto a C30 column. The solvent system consists of pure methanol and MTBE. Initial mobile phase concentration, gradient slope, and flow rate were optimized to reduce analysis times by 60% compared to the existing method while maintaining the minimum desired resolution.

Keywords: Xanthophylls, C_{30} column, Corn extracts

INTRODUCTION

Lutein and β-carotene are the most prominent xanthophylls and carotenoids in human serum and foods. In the 1990s, xanthophylls such as lutein and zeaxanthin became recognized for their health benefits, mainly for the treatment of age related macular degeneration. In addition, there is evidence that these xanthophylls offer protection against some forms of cancer including lung, oral, esophagus/stomach, colon/rectum, breast, prostrate, cervical, and skin.[1] Since humans and animals are unable to biosynthesize carotenoids, they must obtain them from food.[2] There are several methods of producing xanthophylls chemically, e.g., from polyene alcohols[3] or biologically, by expressing them in bacteria or fungi.[4] Xanthophylls may also be extracted from natural xanthophyll rich sources such as corn,[5] green plants, and

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vegetable oils[6] using organic solvents, such as ethanol and hexane. The increasing demand for lutein and zeaxanthin has created a need for a rapid method of analysis of lutein and zeaxanthin.

The most accurate and reproducible method of analyzing xanthophylls is by HPLC. Most carotene separations are conducted with monomeric 5 μm C_{18} spherical particles packed in a 4.6 × 250 mm column. Monomeric phase media are simpler to use and the results are reproducible.[7] Polymeric C_{18} phases, on the other hand, have excellent selectivity for structurally similar carotenoids, e.g., to separate geometric isomers of β-carotene[8] and lutein from zeaxanthin.[9] However, they tend to produce broad peaks with a lot-to-lot reproducibility issue.[7] In general, polymeric C_{18} columns allow for the detection of carotene isomers, while monomeric columns provide for some separation of certain xanthophylls.[10]

At present, the best column for optimum separation of all carotenoids is a polymeric C_{30} column.[11] This column was used earlier in our laboratory to analyze a complex mixture of carotenoids and tocopherols simultaneously from palm oil[12] and for analysis of xanthophylls in corn by Moros et al.[13] The method required analysis times of over 60 minutes per sample, a complex gradient of ternary solvent mixtures, and several sample preparation steps, including extraction with a mixture of butylated hydroxytoluol (BHT) and ethanol (EtOH), boiling, saponification, hexane extraction, evaporation, and resuspension in the mobile phase. This paper describes a simplified version of this method for the rapid analysis of xanthophylls in corn extracts.

EXPERIMENTAL

Reagents

Xanthophyll standards, such as lutein, zeaxanthin, and cryptoxanthin (Extrasynthase Company, Genay, France) were reconstituted to 1 mg/L in pure ethanol obtained from Sigma Aldrich, St. Louis, MO. Commercial lutein was obtained from DSM (formerly Roche Chemicals Ltd) Basel, Switzerland. It was supplied at a concentration of 20% (w/w) in vegetable oil. For these experiments, it was reconstituted in ethanol (200 proof USP grade, Aaper Alcohol and Chemical Co., Shelbyville, KY). The standards solutions were stored at −20°C in glass vials covered with aluminum foil and equilibrated to room temperature prior to use. The mobile phases were HPLC grade methanol (MeOH, Sigma Aldrich, St. Louis, MO), methyl-tertiary-butyl-ether (MTBE, Fisher Scientific, Pittsburgh, PA), and deionized distilled water that was microfiltered with a 0.2 μm Maxi Capsule Filter (Pall Gelman Lab, Ann Arbor, MI). Extraction of xanthophylls from corn was done with ethanol (200 proof USP grade) procured from Aaper Alcohol and Chemical Co. (Shelbyville, KY).

HPLC System

The HPLC system was a Waters HPLC system consisting of a 600E quaternary pump, 2996 PDA photodiode array detector, and the Waters Empower 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 (4.6 mm × 23 mm) containing the same packing material as the C_{30} column was installed ahead of the carotenoid column. A gradient system was used with methanol and MTBE mixed separately or in pure form. Unless otherwise mentioned, the flow rate was 1.0 mL/min, the analysis was done at 25°C and the gradient slope was 1.11% per min. All samples were injected via a 100-μL loop using a 1 mL syringe, unless otherwise mentioned.

Corn Extract

Raw whole corn (yellow dent #2, 14% moisture) was obtained from Anderson Grain Co. (Champaign, IL). The corn was ground using a bench top hammer mill (IKA MF 10.2, IKA Works Inc., Wilmington, NC) with 1 mm pore mesh. Extraction was done with 70% (v/v) aqueous ethanol using a solvent:solids ratio of 4 L aqueous ethanol per kg of ground corn at 50°C with mixing for 30 min. The slurry was then filtered with Whatman No.1 filter paper (11 μm avg. pore diameter). The extract was stored at 40°C until used.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram of xanthophylls standards using the Moros et al. method.[13] The gradient system consisted of two separately mixed mobile phases. Mobile phase A was methanol/MTBE/water (81:15:4) and

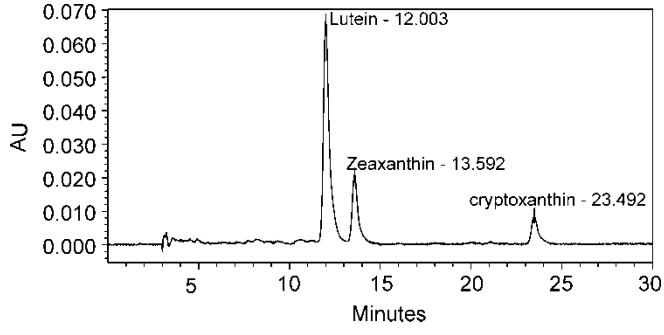

Figure 1. HPLC chromatogram of xanthophylls standards using the Moros et al. method.

mobile phase B was methanol/MTBE (9:91). The gradient was 100% of A and 0%B to 50%A and 50%B in 45 min, followed by 100%B for 15 min, and finally re-equilibration to 100%A for 25 min (equivalent to about 5 column volumes). The effective time required for one analysis was 85 min, which includes the gradient, cleaning, and re-equilibration times.

The modification of the Moros et al. method was done in five steps addressing the following issues: the gradient, the mobile phase, initial solvent composition, gradient rate, flow rate, and sample preparation.

Gradient

The retention time for the last peak (β-cryptoxanthin) was 23 min and, yet, the gradient in Moros' method was 45 min. By modifying the gradient from an initial value of 100%A/0%B to 67%A/33%B in 30 min, followed by 100%B for 15 min, and re-equilibration to 100%A for 25 min, the resolution of the isomers remained unchanged, while the analysis time was reduced by 9 min per cycle (Figure 2). The process was repeated with only lutein and zeaxanthin with a gradient 100%A/0%B to 80%A/20%B in 16 min, followed by 100%B for 15 min, and re-equilibration to 100%A for 25 min. This reduced total analysis time for the three isomers to 70 min and for lutein and zeaxanthin to 56 min.

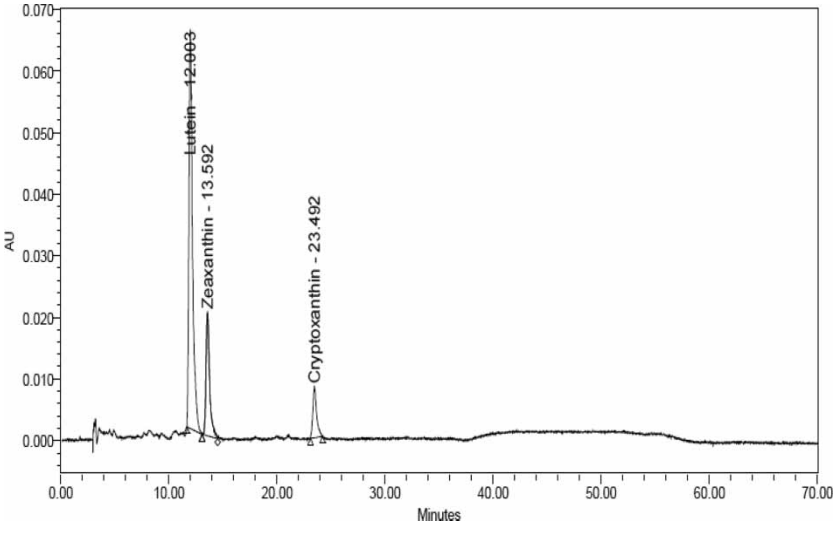

Figure 2. Effect of changing gradient table to reduce run time of xanthophylls standards. Gradient: 100%A to 66.7%A/33.3%B in 30 min, followed by 100%B for 15 min, and re-equilibration to 100%A for 25 min. Mobile phase A is the solvent mixture methanol/MTBE/water (81:15:4) and mobile phase B was methanol/MTBE (9:91). The flow rate was 1 mL/min at 25°C and injection volume was 100 μL.

Mobile Phase

The original mobile phase consisted of three solvent mixtures, which makes the method complicated, enhances demixing, and results in different evaporation rates, causing variation in the retention times.[7] This was simplified to three pure solvents (methanol, MTBE, and water, designated as solvents X, Y, and Z, respectively). This was possible only because a quaternary pump was used in our HPLC system. The new gradient was 81%X/15%Y/4%Z to 59.4%X/37.8%Y/2.8%Z in 27 min, followed by 9%X/91%Y for 15 min, and re-equilibration to 81%X/15%Y/4%Z for 25 min. Figure 3(a) shows no significant change by using single solvents; in fact, there was a slight improvement in resolution of the two isomers.

However, even this simplified mobile phase had three solvents. The next objective was to eliminate water from the gradient system. In order to maintain the polarity of the mobile phase, the amount of methanol was increased using Snyder's polarity index.[14] The polarities of methanol, MTBE, and water are 6.6, 2.5, and 9, respectively. Thus, the Snyder polarity index (P′) of the solvent mixture of 81:15:4 methanol:MTBE:water was 6.1. To retain the same polarity index without water, methanol was increased from 81% to 90%. The new gradient with mobile phases X and Y as methanol and MTBE, respectively, was as follows: 90%X/10%Y to 57%X/43%Y in 30 min, followed by 9%X/91%Y for 15 min, and re-equilibration to 90%X/10%Y for 25 min. As seen in Figure 3(b), this modification reduced the run time although it reduced the resolution; there was still a baseline separation for the two xanthophylls isomers.

Flow Rate

Several flow rates between 0.5 mL/min and 1.25 mL/min were studied.[15] The best flow rate was 1 mL/min since the peaks overlapped at higher flow rates.

Initial Solvent Ratio

Several initial ratios of MeOH:MTBE were evaluated with the same gradient slope (1.11%/min), flow rate (1 mL/min), and run time of 15 min. Increasing the non-polar nature of the initial solvent caused a reduction in resolution, e.g., peaks that were separate at 90:10 MeOH:MTBE overlapped at 75:25 MeOH:MTBE. The best initial solvent ratio in terms of resolution was 90:10 as shown in Figure 4.

Gradient Slope

Each compound elutes when the polarity of the mobile phase offers an affinity greater than the stationary phase. An increase in the gradient slope decreases

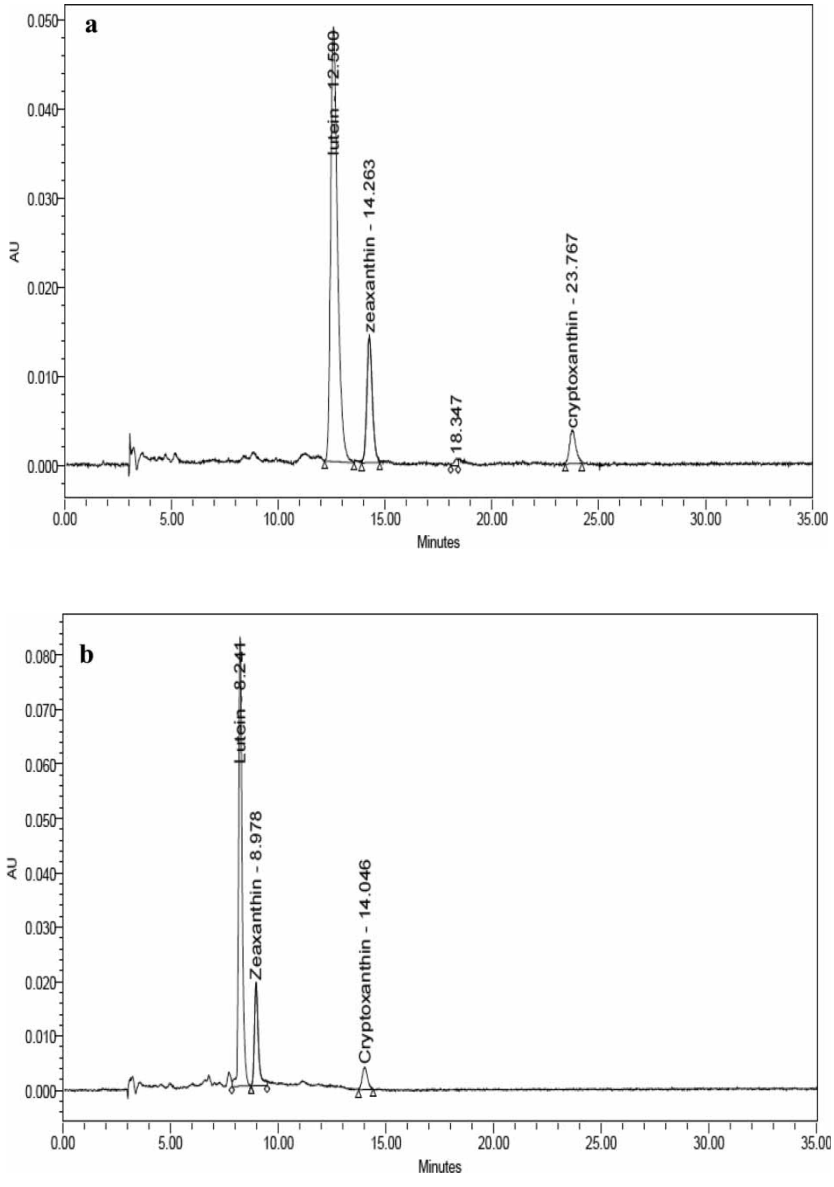

Figure 3. Effect of mobile phase on xanthophylls standards. (a) Three pure solvents: Gradient was 81%X/15%Y/4% Z to 57%X/40.3%Y/2.7%Z in 30 min, followed by 9%X/91%Y for 15 min, and re-equilibration to 81%X/15%Y/4%Z for 25 min where X, Y, Z are MeOH, MTBE, and water, respectively. (b) Two pure solvents (without water): Gradient was 90%X/10%Y to 57%X/43%Y in 30 min, followed by 9%X/91%Y for 15 min, and re-equilibration to 90%X/10%Y for 25 min where X and Y are MeOH and MTBE, respectively. The flow rate in each case was 1 mL/min at 25°C and 100 μL injection.

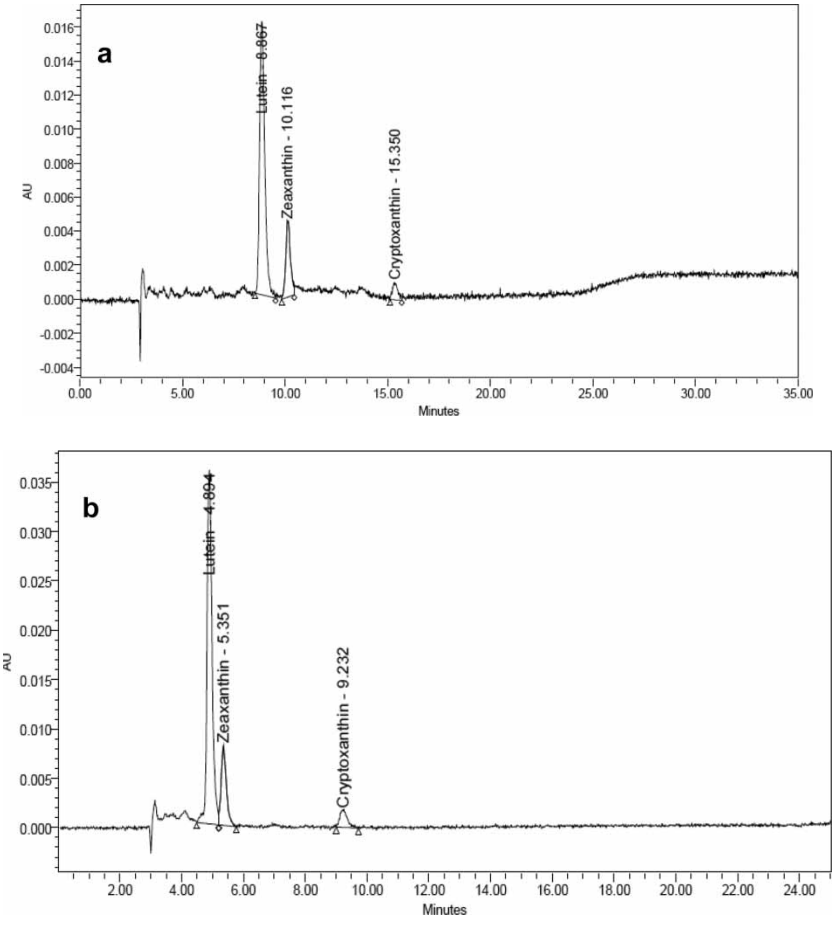

Figure 4. Effect of initial solvent ratio. (a) 90:10 MeOH:MTBE, (b) 75:25 MeOH:MTBE. The gradient slope was 1.11%/min, with a flow rate of 1 mL/min at 25°C and an injection size of 100 μL.

the polarity in the column faster, thus reducing analysis time, but it also reduces the resolution. Several gradients were evaluated with the initial solvent ratio of 90:10 MeOH:MTBE, a flow rate of 1 mL/min, and a run time of 25 min.[15] The best results were obtained with a gradient slope of 2%/min.

Sample Preparation

Xanthophylls are extremely unstable to heat, light, oxidation, and acids,[16] and this has to be taken into account during sample preparation and analysis. With corn extracts, Moros et al.[13] proposed boiling and saponification to

eliminate oils and hydrolyze carotenoid esters. Work in our laboratory has shown that adding water to the extraction solvent (ethanol) would eliminate coextraction of oil[17] and, thus, the saponification and boiling steps could be eliminated. Moros et al. also used hexane extraction followed by evaporation

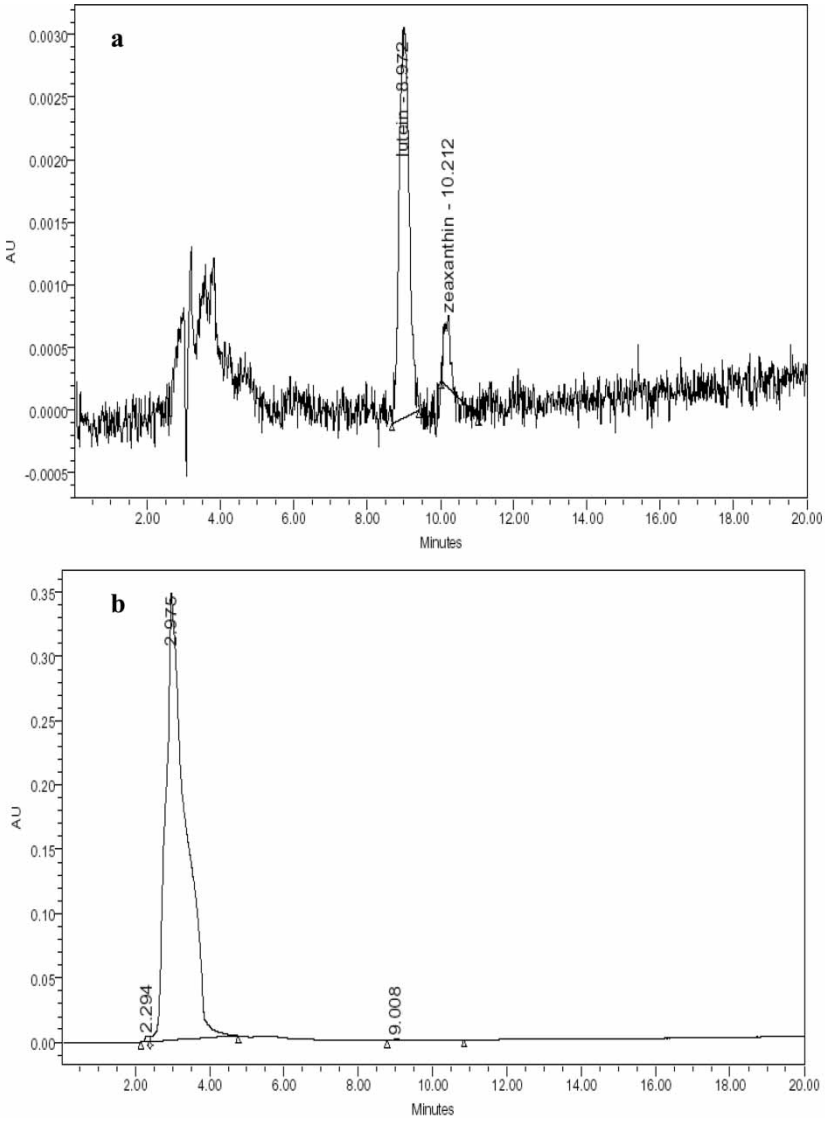

Figure 5. HPLC of corn extract using modified method. Initial solvent ratio was 90:10 MeOH:MTBE, gradient was 2% per min; flow rate was 1 mL/min at 25°C, and injection size of 100 μL. Detection at (a) 450 nm, and (b) 280 nm.

and resuspension to eliminate other impurities, such as the ethanol-soluble protein zein. However, the solubility of xanthophylls is very low in hexane[18] and the hexane extract may not represent the actual xanthophylls concentration in the sample. In addition, since zein and impurities do not interact with the column and elute in the void volume, this step could also be eliminated. Figure 5 shows the analysis of an ethanol extract of corn that was only microfiltered prior to injection into the column. Figure 5a shows xanthophyll levels with the PDA detector set at 450 nm, while Figure 5b shows measurements at 280 nm to measure protein and other solids.

However, the low concentration of xanthophylls in the extract results in a low detector response with relatively high baseline noise (Figure 5a). The noise is probably due to polar impurities in the sample, which was eliminated by the hexane extraction step in the Moros et al. method. Concentration of xanthophylls in raw corn is 20–25 ppm[13] and 2–10 ppm in the ethanol extracts, depending on the solvent:solids ratio. These low levels in the extract result in low accuracy and reproducibility. This was demonstrated by multiple injections and analyses. Standard solutions containing lutein concentrations of 0.5–5000 ppm were injected at least five times each into the HPLC and the corresponding peak areas were plotted against concentration (Figure 6). The standard solutions show good linearity within the concentration range examined, as shown by the high correlation coefficient. Table 1 shows the

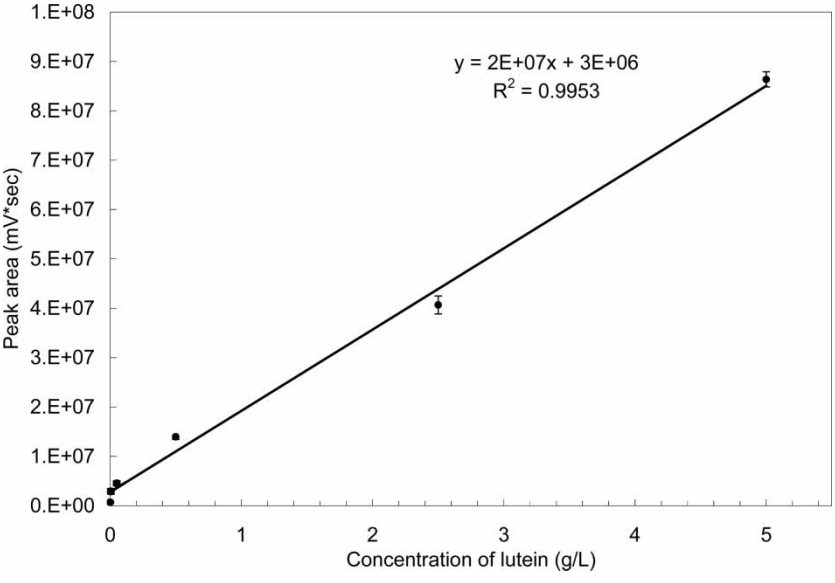

Figure 6. Calibration curve for commercial lutein dissolved in pure ethanol. Initial solvent ratio was 90:10 MeOH:MTBE, gradient was 2% per min; flow rate was 1 mL/min at 25°C and injection size was 20 μL. Bar represents mean ± one standard deviation.

Table 1. Statistical analysis of the data obtained in the calibration curve for commercial lutein standard (Figure 6)

Lutein concentration		Mean peak area (mV*s)	Standard deviation	Relative standard deviation (RSD, %)
Ppm	g/L			
0.5	0.0005	693,381	67,039	9.6
5	0.005	2,910,285	552,792	18.9
50	0.05	4,541,036	473,905	10.4
500	0.5	13,972,354	535,405	3.8
2500	2.5	40,668,725	1,803,078	4.4
5000	5	86,359,753	1,534,613	1.7

statistical analysis of the calibration curves. The relative standard deviation (RSD) is high for concentrations of lutein lower than 500 ppm, and decreases at higher lutein levels to less than 5% RSD.

With corn extracts, reproducibility was estimated by diluting the extracts to various concentrations using 70% aqueous ethanol and injecting the samples into the column. Each analysis was performed with three injections of each sample. As shown in Figure 7, the xanthophylls in corn extracts showed good linearity ($R^2 = 0.9719$) within the concentration range examined.

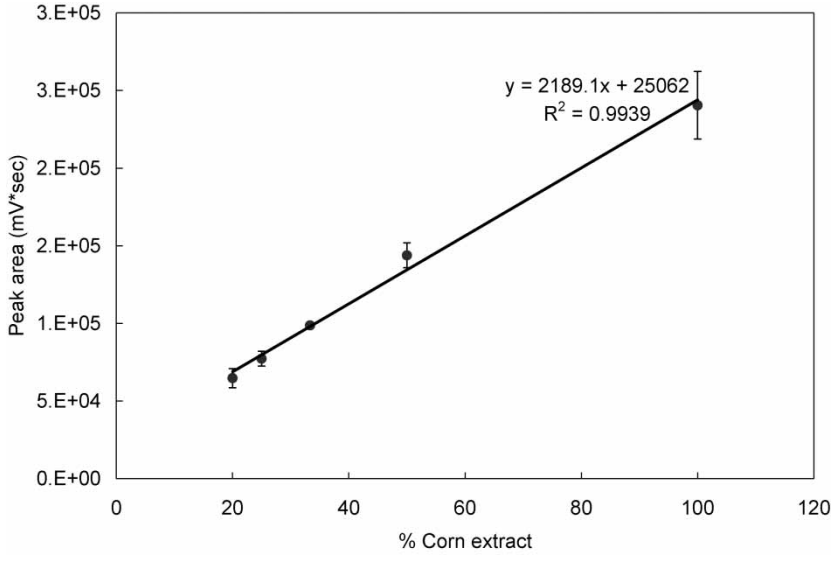

Figure 7. Calibration curve for lutein in corn extracts. Initial solvent ratio was 90:10 MeOH:MTBE, gradient was 2% per min; flow rate was 1 mL/min at 25°C and injection size was 20 μL. Bar represents mean ± one standard deviation.

CONCLUSIONS

A rapid, simple, HPLC method for analyzing xanthophylls (lutein and zeaxanthin) in corn has been developed using the YMC30 column using an initial solvent ratio of 90:10 MeOH:MTBE, flow rate of 1 mL/min at 25°C, gradient slope of 2% per min, and 100 μL injections. The method does not require sample preparation other than extraction with 70% ethanol and microfiltration. The analysis takes 15 min with another 15 min for cleaning and equilibration. This method is promising for rapid qualitative analysis of xanthophylls at the low concentrations in ethanol extracts. However, to improve accuracy, it is recommended that the extract be evaporated and reconstituted in the mobile phase, as done by Moros et al.,[13] to increase the concentration of lutein and zeaxanthin in the injected sample.

ACKNOWLEDGMENTS

This work was supported by the Illinois Corn Marketing Board, Bloomington, IL, and the U. S. Department of Agriculture under the NRICGP program (CSREES-NRI grant # 2004-35503-14116). Financial support in the form of scholarships to AK was provided by AACC International, St. Paul, MN and the Department of Food Science & Human Nutrition, University of Illinois at Urbana-Champaign. The University of Illinois Research Board provided an equipment research grant.

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Received August 27, 2006
Accepted November 2, 2006
Manuscript 6392