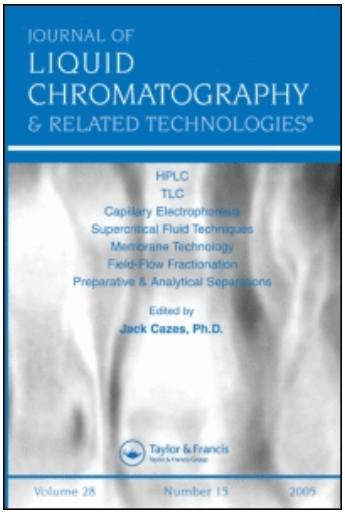

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 17 November 1999

To cite this Article Kurilich, Anne C. and Juvik, John A.(1999) 'SIMULTANEOUS QUANTIFICATION OF CAROTENOIDS AND TOCOPHEROLS IN CORN KERNEL EXTRACTS BY HPLC', Journal of Liquid Chromatography & Related Technologies, 22: 19, 2925 — 2934
To link to this Article: DOI: 10.1081/JLC-100102068
URL: http://dx.doi.org/10.1081/JLC-100102068

J. LIQ. CHROM. & REL. TECHNOL., 22(19), 2925–2934 (1999)

SIMULTANEOUS QUANTIFICATION OF CAROTENOIDS AND TOCOPHEROLS IN CORN KERNEL EXTRACTS BY HPLC

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ABSTRACT

An isocratic high performance liquid chromatographic (HPLC) method with run times of less than 22 min has been developed for simultaneous analysis of carotenoids and tocopherols in corn. The reverse phase system utilizes a nonaqueous mobile phase in combination with a polymeric C_{18} column coupled to a monomeric C_{18} column to separate lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and the α-, δ- and γ- forms of tocopherol. The detector was a multi-channel UV-VIS. One channel was used to monitor carotenoid absorption at 450 nm while another monitored tocopherols at 290 nm.

Retention times and spiked tests with known standards validated peak identification. Linear regressions of external standards generated coefficients of determination (R^2) that were in the ninety-ninth percentile for all compounds tested. Using the same samples, concentrations generated from our protocol and compared with results from other laboratories suggest that this procedure is relatively accurate.

INTRODUCTION

Carotenoids and tocopherols can function both as pro-vitamins/vitamins (vitamin A and E) and as antioxidants that can scavenge free radicals in biological systems. Free radicals cause damage to cells, tissues, and DNA, which may lead to the induction of cancer.[1] Recent studies have shown that the antioxidant carotenoids and tocopherols may have the potential to reduce the risk of many chronic diseases such as cancer and cardiovascular disease.[2-5] Carotenoids and tocopherols are found naturally in many vegetables. Quantification of these compounds in our food supply will play an important role in determining their potential benefits for human health.

Several high performance liquid chromatographic (HPLC) methods are available for analyzing carotenoids and tocopherols separately.[6-9] However, a method allowing simultaneous analysis could simplify the procedure, shorten analysis time, and reduce operating costs. Such procedures have been developed for use in determining carotenoid and tocopherol composition in human and rat serum samples,[10-11] but a similar protocol for analysis of plant tissue is lacking. Weber[6] developed a procedure for simultaneous extraction of these lipid soluble compounds. Here, we have modified and combined two separate protocols to allow for simultaneous extraction, separation, and quantification of several carotenoids and tocopherols.

EXPERIMENTAL

Plant Material

Seed of five sweet corn inbred lines (C68, IL27a, IL442a, IL618b, and IL677a) were sown in field plots in a randomized complete block design with three replicates at the University of Illinois South Farm. Twenty days after self-pollination 4-6 ears of each genotype were harvested in each replicate block. Ears were immediately frozen in liquid nitrogen and stored in freezer bags at -80°C. Fifty kernels were removed from each ear, bulked into sample bags, and freeze-dried to remove moisture. Freeze-dried corn samples were ground into powder using a coffee grinder and stored at -20°C until extraction.

Standard Preparation

Lutein, zeaxanthin, β-cryptoxanthin, and β-carotene standards were obtained from Extrasynthese (Genay, France). Standards for α-carotene, and α-, δ-, and γ-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Standards of α- and β-carotene were prepared in hexane, while tocopherols, lutein, zeaxanthin, and β-cryptoxanthin were prepared in absolute ethanol.

Actual concentrations of stock solutions were determined spectrophotometrically using spectral absorption coefficients: lutein = 2765 at 445 nm, zeaxanthin = 2416 at 452 nm, β-cryptoxanthin = 2486 at 452 nm, α-carotene = 2800 at 444 nm, β-carotene = 2592 at 452 nm, α-tocopherol = 75.8 at 292 nm, γ-tocopherol = 91.4 at 298 nm, and δ-tocopherol = 91.2 at 297 nm (12). Three sets of working standards (lutein and zeaxanthin; β-cryptoxanthin, α- and β-carotene; α-, δ-, and γ-tocopherol) at five concentrations were prepared, dried down in a vacuum concentrator and reconstituted in acetonitrile: methanol: methylene chloride (45:20:35 v/v/v). Concentration ranges for carotenoid standards were lutein = 0.116 – 150 μg/mL; zeaxanthin = 0.069 – 90 μg/mL; β-cryptoxanthin = 0.022 – 14 μg/mL; α-carotene = 0.013 – 8 μg/mL; and β-carotene = 0.016 – 10 μg/mL. Ranges of tocopherol standards were α- and δ-tocopherol = 0.4 – 50 μg/mL; γ-tocopherol = 1.44 – 180 μg/mL.

Sample Extraction Procedures

The following extraction procedure is a modification of a method developed by Weber.[6] All sample preparations and extractions were performed under gold fluorescent lights. Freeze-dried tissue samples of 600 mg were used for extraction of corn samples. Ethanol (6 mL) containing 0.1% butylated hydroxytoluene (BHT) was added to each sample before placing in an 85°C water bath for 5 min. After removal from the water bath, 120 μL 80% potassium hydroxide (KOH) was added, samples vortexed for 20 sec and returned to the water bath for a 10 min saponification. All samples were vortexed once more during saponification. Upon removal they were immediately placed in an ice bath where 3 mL cold deionized distilled H_2O was added. Each sample then received 3 mL hexane and was vortexed and centrifuged for 10 min at 1200 x g. The upper layer was pipetted into a separate test tube and the pellet was re-extracted twice more using hexane. The combined upper layers were washed with 3 mL deionized distilled H_2O, vortexed, and centrifuged for 10 min, before the hexane fraction was again removed to another test tube. The hexane fraction was dried down in a vacuum evaporator and samples were reconstituted in 200 μL of acetonitrile: methanol: methylene chloride (45:20:35 v/v/v).

HPLC System

The HPLC system consisted of an ERMA Optima LTD ERC 3510 degasser (Anspec Co., Ann Arbor, MI), a Waters 510 pump, 731a auto-injector, and a 490E multi-wavelength UV-Vis detector (Waters Chromatography, Milford, MA). Carotenoids were detected at 450 nm, while tocopherols were detected at 290 nm. Sensitivity was set at 0.08 absorbance units full scale (AUFS). Data were collected and processed using Waters Millennium 2010

software (Waters Chromatography, Milford, MA). The columns consisted of a polymeric Vydac 201TP54 C_{18} reverse phase (5 μm, 4.6 x 150 mm) column (Separations Group, Hesperia, CA) connected to a monomeric Waters Nova-Pak C_{18} reverse phase (4 μm, 3.9 x 150 mm) column (Waters Chromatography, Milford, MA). The columns were protected by an Adsorbosphere C_{18} reverse phase (5 μm, 4.6 x 7.5 mm) guard column (Alltech Assoc., Deerfield, IL). The mobile phase consisted of acetonitrile: methanol: methylene chloride (75:20:5 v/v/v), containing 0.05% triethylamine and 0.1% BHT[7] with a flow rate of 1.8 mL/min. Thirty μL of each sample extract were injected into the HPLC.

Analytical Evaluation

Three experiments were run to test the accuracy and reproducibility of this procedure. Sweet corn samples were spiked with a known amount of individual standard, then extracted and run to obtain recovery values for each compound. One corn genotype (C68) was randomly chosen to investigate reproducibility between separate extractions of the same tissue sample and between aliquots of the same extraction. Tissue was extracted once and then three aliquots were analyzed to estimate within extract variability. Three separate extractions of tissue of the same sweet corn sample were run to evaluate within sample variability. Finally, samples of bulked kernel tissue from each of the field replicates were assayed to estimate variability between samples of the same genotype.

Analytical Comparisons

Samples of two genotypes (C68 and IL618b) were sent to the ARS/USDA Phytonutrient Lab in Beltsville, MD supervised by Dr. Beverly Clevidence for analysis of carotenoids using standard AOAC methods to compare results with our procedure. These same samples were also sent to Maria S. Sapuntzakis, Senior Research Specialist in the Health Sciences at the University of Illinois at Chicago for further comparison of carotenoid and tocopherol content.

Statistical Analyses

Sample concentrations were calculated by comparing peak area of samples to peak area of standards. Recovery values for individual compounds were obtained by comparing concentrations of spiked to unspiked samples. Regression of peak area on standards of known concentrations was performed to obtain the coefficient of determination (R^2) for each compound. The coefficient of determination represents the proportion of the total treatment sums of squares accounted for by the regression.[13]

Means and standard deviations were calculated to estimate reproducibility between separate extractions of the same tissue sample, aliquots of the same extraction, and tissue sample variation.

Analysis of variance and mean separation was conducted to test for significant differences in compound concentrations between the samples analyzed at the University of Illinois, UIC, and the USDA lab.

RESULTS AND DISCUSSION

Analytical Procedure

An analytical procedure that is both fast and accurate is needed to simultaneously quantify individual carotenoids and tocopherols. Both normal and reverse phase systems have been used to quantify carotenoids and tocopherols separately,[7,9] although the use of normal phase columns often resulted in co-elution of major carotenes.[6] C_{18} reverse phase columns have been used to separate xanthophylls or carotenes, but different packing materials or solvent systems are required for each class of compounds.[14]

Hart and Scott[7] developed methodology that employed a two column system (Alltech ODS2 and Vydac 201TP54) as a means of circumventing this problem. The two-column combination successfully eluted both xanthophylls and carotenes. When tocopherols were added to the mixture, they co-eluted with the xanthophylls. Lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene were resolved at 450 nm while δ-tocopherol, γ-tocopherol and α-tocopherol were resolved at 290 nm.

Although no tocopherols are detected at 450 nm, carotenoids will absorb at 290 nm and interfere with quantification of the tocopherols. When the Alltech ODS2 column was exchanged for a Waters Nova-Pak, all eight compounds eluted separately and could be quantified independently (Figure 1).

Analytical Evaluation

Identification of each compound was validated using retention times of known standards and spiking samples with individual standards before extraction. Figure 1 shows carotenoid and tocopherol chromatograms of a corn sample. The xanthophylls and tocopherols eluted within 10 min, while the carotenes closer to 20 min, which is similar to isocratic procedures used for separate analysis of these compounds.[6,7,10]

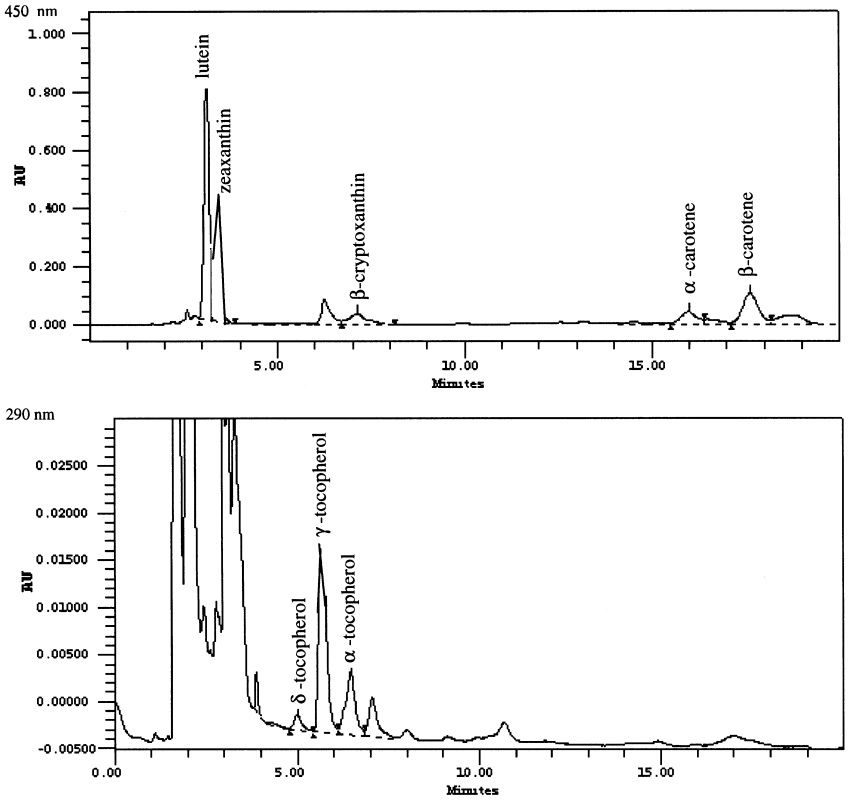

Figure 1. Carotenoid chromatogram (top) at 450 nm. Peaks have been identified as lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene. Tocopherol chromatogram (bottom) at 290 nm. Peaks are δ-tocopherol, γ-tocopherol, and α-tocopherol.

Recovery values (Table 1) ranged from 85% to 122% indicating that the extraction procedure is effective. These values are slightly higher than those reported by Weber[6] for β-carotene and γ-tocopherol, and very similar to those reported by Chuang et al.[10] for both carotenoids and tocopherols. Regression analysis of five external standards at a broad range of concentrations generated coefficients of determination that were greater than 99.9% for all compounds (Table 1).

Table 1

Recovery Values, Standard Deviations, and Coefficients of Determination (R^2) for Individual Carotenoids and Tocopherols in Sweet Corn Samples

Compound	N	% Recovery	No. of Standards	R^2 (%)
Lutein	2	99 ± 18	5	99.9
Zeaxanthin	2	85 ± 6	5	99.9
β-cryptoxanthin	2	116 ± 17	5	99.9
α-carotene	2	119 ± 24	5	99.9
β-carotene	2	122 ± 27	5	99.9
α-tocopherol	2	103 ± 16	5	99.9
δ-tocopherol	2	85 ± 2	5	99.9
γ-tocopherol	2	115 ± 15	5	99.9

Table 2

Reproducibility Results Among Aliquots of the Same Extraction (C68) and Different Extracts of the Same Tissue Sample (C68)*

	N	Same Extract	Different Extracts
Lutein	3	13.34 ± 0.94	14.17 ± 1.32
Zeaxanthin	3	7.73 ± 0.40	7.37 ± 0.78
β-cryptoxanthin	3	0.85 ± 0.05	1.02 ± 0.10
α-carotene	3	0.30 ± 0.03	0.26 ± 0.01
β–carotene	3	7.65 ± 0.51	8.05 ± 0.27
α-tocopherol	3	12.42 ± 1.25	12.37 ± 1.82
δ-tocopherol	3	0.80 ± 0.08	0.80 ± 0.10
γ-tocopherol	3	14.57 ± 1.74	14.18 1.41

* Mean concentrations and standard deviations are expressed in μg/g dry weight.

One corn genotype was randomly chosen to test reproducibility between separate extractions of the same tissue sample and between aliquots of the same extraction. The results (Table 2) show that the variation attributed to the extraction procedure and the sample replicates is minimal, indicating that reproducibility was high.

Table 3

Comparison of Data from Three Methods; Means and Standard Deviations of Sweet Corn Genotypes (μg/g Dry Weight) Harvested at 20 DAP

Simultaneous Method

Genotype	N	Lutein	Zeaxanthin	β-Cryptoxanthin	α-Carotene	β–Carotene	α-Tocopherol	δ-tocopherol	γ-Tocopherol
IL27a	3	0.11 ± 0.04	0.09 ± 0.01	0.08 ± 0.01	0.03 ± 0.00	0.14 ± 0.02	3.07 ± 1.23	0.79 ± 0.10	12.54 ± 0.12
IL442a	3	2.00 ± 0.11	1.61 ± 0.04	0.62 ± 0.05	0.09 ± 0.01	0.41 ± 0.15	5.13 ± 1.88	1.33 ± 0.31	18.35 ± 6.50
IL677a	3	1.61 ± 0.04	1.77 ± 0.08	0.34 ± 0.06	0.10 ± 0.02	0.39 ± 0.07	5.78 ± 0.90	1.29 ± 0.42	19.29 ± 2.08
IL618b	3	7.37 ± 2.88 a	2.23 ± 0.82 a	0.29 ± 0.04 a	0.14 ± 0.09 a	1.46 ± 1.08 a	6.55 ± 2.52 a	1.73 ± 0.49 a	34.48 ± 9.55 a
C68	3	14.51 ± 1.56 a	7.52 ± 0.17 a	1.16 ± 0.06 a	0.26 ± 0.01 a	7.97 ± 0.35 a	10.00 ± 0191 a	1.17 ± 0.13	13.55 ± 1.68 a
AOAC Method (USDA Phytonutrient Lab)									
IL618b	4	8.35 ± 0.78 a	NA	0.30 ± 0.09 a	0.47 ± 0.14 b	3.28 ± 0.46 b	NA	NA	NA
C68	2	17.36 ± 0.33 a	NA	1.01 ± 0.01 a	0.86 ± 0.02 b	9.17 ± 0.82 b	NA	NA	NA
University of Illinois - Chicago									
IL681b	2	7.18 ± 0.10 a	3.21 ± 0.02 a	0.44 ± 0.01 a	0.29 ± 0.04 ab	2.29 ± 0.03 ab	ND	NA	22.40 ± 0.50 a
C68	2	16.4 ± 0.08 a	9.25 ± 0.03 a	2.42 ± 0.02 a	0.65 ± 0.02 ab	8.98 ± 0.08 ab	11.70 ± 0.01 a	NA	13.50 ±0.01 a

NA = not available. ND = not detected. Means with the same letter are not significantly different at $p > 0.05$.

Results from this study showed a 7-8% variation in lutein and a 6-7% variation in β-carotene between three extractions of the same sample, which was lower or comparable to the 11-43% (lutein) and 5-7% (β-carotene) variation reported by Hart and Scott.[7]

Table 3 shows the results from the five corn lines sampled. Generally, the reproducibility within the same sample from three separate replications was very good. The data presented here is comparable to values reported by Lee et al.[15] for α-carotene, β-carotene and β-cryptoxanthin concentrations in sweet corn. Results from the standard AOAC methods run at the ARS/USDA Phytonutrient Lab and from the University of Illinois at Chicago (UIC) (Department of Human Nutrition and Dietetics) are also shown in Table 3. Concentrations for xanthophylls were statistically non-significant between methods. Carotene concentrations were not significantly different from UIC results but were significantly lower than those generated by the USDA lab. Mean tocopherol concentrations for the two genotypes assayed by our method and at UIC were not significantly different.

The procedure described above appears to provide an efficient, relatively accurate, and simultaneous method of quantifying carotenoids and tocopherols in corn kernels. It is less time consuming then separate analysis and therefore, more cost efficient. This procedure was developed to survey sweet corn germplasm for variation in carotenoid and tocopherol content and identify genotypes suitable for use in vegetable crop genetic improvement programs. Studies are currently under way to investigate modifying this procedure for quantification of these compounds in other vegetables.

ACKNOWLEDGMENTS

Financial support of this project was sponsored through grants 97I-54 and 98E-57 from the Illinois Council for Agricultural Research (C-FAR) and the University of Illinois Agricultural Experiment Station Strategic Research Initiative. The authors wish to thank Dr. Beverly Clevidence and Gene Wiley from the ARS/USDA Phytonutrient Lab, Maria S. Sapuntzakis, Senior Research Specialist in the Health Sciences at the University of Illinois at Chicago, and Jeff Wong and Dr. Torbert Rocheford from the Crop Sciences Department at the University of Illinois-Urbana for their contributions and assistance.

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Received March 8, 1999
Accepted March 31, 1999
Manuscript 5027

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