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Fast Quantitative Method for the Analysis of Carotenoids in Transgenic Maize

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ABSTRACT: A fast method was developed to determine carotenoid content in transgenic maize seeds. The analysis was carried out using an ultrahigh-pressure liquid chromatograph coupled to a photodiode array detector and a mass spectrometer (UHPLC-PDA-MS/MS). Sixteen carotenoid pigments were detected and quantified in <13 min. In addition, it was possible to obtain good resolution of both polar xanthophylls and nonpolar carotenes. The method exhibited (a) a high degree of repeatability (%RSD < 13%), (b) linear calibration curves (R^2 > 0.9952), (c) satisfactory recoveries for most of the pigments (between 82 and 108%), and (d) low detection (from 0.02 to 0.07 μg/mL) and quantification limits (from 0.05 to 0.20 μg/mL) (LOD and LOQ, respectively). The methodology was applied to the analysis of transgenic maize lines TM1, TM2, and TM3, expressing several carotenogenic genes.

KEYWORDS: *maize, carotenoids, ultrahigh-pressure liquid chromatography (UHPLC)*

INTRODUCTION

Maize is one of the most important agronomic crops in the world. This cereal and rice are the staple diet of >75% of the human population.[1] In addition, maize is also widely used in animal feeds and as a resource for many industrial and commercial products.[2,3] Yellow maize contains both provitamin A and non-provitamin A carotenoids with potential health benefits to humans.[4−6] Carotenoids are hydrophobic molecules with little or no solubility in water. These pigments are isoprenoid compounds, biosynthesized by tail-to-tail linkage of two C20 geranylgeranyl diphosphate molecules. Carotenoids that contain one or more oxygen functions are known as xanthophylls.[7] Efforts to increase concentrations of these important micronutrients in maize have been reported recently.[8] For example, by using combinatorial nuclear transformation, Zhu et al.[9] were able to obtain transgenic kernels with high levels of β-carotene (57.35 μg/g DW). In addition, the same transgenic plants had increased levels of lycopene, zeaxanthin, lutein, and astaxanthin, all molecules of nutritional and industrial importance. Another significant advance in this area was achieved by Naqvi et al.,[10] who obtained transgenic maize seeds containing 169-, 23-, and 112-fold more β-carotene, lycopene, and zeaxanthin, respectively, than the corresponding wild type plants.

Humans need to acquire carotenoids through their diet because they are not able to synthesize them de novo. Although >700 carotenoids have been described in nature, not all natural sources are present in our normal diet. It is estimated that we have access to only about 40 carotenoids that can be absorbed, metabolized, and/or used in our bodies.[11] However, nearly 20 carotenoids of those 40 have been identified in human blood and tissues.[12] Close to 90% of the carotenoids in the diet and human body is represented by α- and β-carotene, lycopene, β-cryptoxanthin, zeaxanthin, and lutein, which are regularly present in food.[12] The intake of carotenoids in diet is associated with a lower risk of developing some diseases such as cancer, heart disease, and macular degeneration.[13,14] Consumption of ketocarotenoids, most notably astaxanthin, is also increasingly associated with a range of health benefits. Some evidence suggests astaxanthin is a potential therapeutic agent for the treatment of oxidative stress, inflammation, and cardiovascular diseases in humans and animals.[4,15−17] Ketocarotenoids are currently being used as feed additives for the aquaculture and poultry industries.[18] These pigments are responsible for the attractive pink and red colors of the feathers and skin of many birds (e.g., flamingo, scarlet ibis, and roseate spoonbill) and the shells of lobster, shrimp, krill, crabs, and other crustaceans.[15] Ketocarotenoids are synthesized by certain bacteria, several fungi, some green algae, and a few species of the flowering plant genus *Adonis*.[17−19] The majority of the demand for astaxanthin is met by chemical synthesis, but natural sources are becoming more important.[15,17]

HPLC using absorption detection technique is currently the most common chromatographic method for the analysis of carotenoids.[20] The recent introduction of ultrahigh-pressure liquid chromatography (UHPLC) offers several advantages over conventional high-pressure liquid chromatography

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(HPLC), such as faster analyses, narrower peaks, and greater sensitivity[21] through the utilization of columns packed with smaller diameter particles (1.7 μm) that can withstand high backpressures compared to the conventional HPLC instrumentation. The reliable and reproducible quantification of plant carotenoids is a complex task. Extensive research has been carried out detailing the precautions and steps to be taken during their analysis.[22−24]

The application of MS to carotenoid analysis has been a significant step forward with regard to classical carotenoid analysis based on the use of spectrophotometric ultraviolet−visible (UV−vis) techniques. Tandem mass spectrometry (MS/MS) offers added selectivity and specificity to the simple LC-MS systems.[21] For example, van Breemen et al.[25] observed that lycopene, γ-carotene, β-carotene, and α-carotene produced the molecular radical ion at m/z 536 during FAB ionization in positive ion mode. However, during collisionally activated dissociation (CAD) only the molecular ion of α-carotene formed unique fragment ions at m/z 388 and 480, corresponding to $[M - 148]^{\bullet+}$ and $[M - 56]^{\bullet+}$. Similarly, Fang et al.[26] observed that the transition 536 → 467 was unique for lycopene using APCI in negative ion mode; consequently, this transition was used to quantify this pigment and distinguish it from its structural isomers β-carotene and α-carotene. Thus, those ions arising directly from the precursor ions produce a fingerprint pattern specific to the compound under investigation.

The aim of this paper is to describe a novel and fast chromatographic method for the analysis of carotenoids in transgenic maize seeds by UHPLC-PDA-MS/MS. In addition, we assessed the reliability of the method by determining basic validation parameters including relative recovery, accuracy, precision, linearity, and detection and quantification limits (LOD and LOQ, respectively).

MATERIALS AND METHODS

Chemicals. β-Carotene, lycopene, lutein, β-cryptoxanthin, astaxanthin, and β-apo-8′-carotenal were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Canthaxanthin and zeaxanthin were acquired from Fluka (Buchs SG, Switzerland). Phytoene and violaxanthin were purchased from Carotenature (Lupsingen, Switzerland). Methanol (MeOH), ethyl acetate, diethyl ether, acetonitrile (ACN), and acetone (HPLC grade purity) were acquired from J. T. Baker (Deventer, The Netherlands). Water was prepared using a Milli-Q reagent water system.

Plant Material. Four maize (*Zea mays*) plants were used: M37W, a South African elite white inbred, and transgenic maize lines TM1, TM2, and TM3, expressing several carotenogenic genes. These plants were generated by combinatorial nuclear transformation, as reported in Zhu et al.[9]

Carotenoid Extraction. The extraction procedure was carried out according to Rivera et al.[22]

Chromatographic Analysis. UHPLC analysis was carried out using an Acquity Ultra Performance LC system linked to a PDA 2996 detector (Waters, Milford, MA, USA). Mass detection was carried out using an Acquity TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). MassLynx software version 4.1 (Waters) was used to control the instruments and also for data acquisition and processing. UHPLC separations were performed on a reversed-phase column Acquity UPLC C18 BEH 130 Å, 1.7 μm, 2.1 × 100 mm (Waters). The mobile phase consisted of solvent A, ACN/MeOH 7:3, v/v, and solvent B, water 100%. The gradient program used is shown in Table 1. The column and sample temperatures were set at 32 and 25 °C, respectively. Injection volume was 5 μL.

Table 1. Gradient Profile Used in the Separation of Carotenoids by UHPLC

time (min)	flow rate (mL/min)	A (%) ACN/MeOH 7:3, v/v	B (%) water	curve
initial	0.4	80	20	linear
2.0	0.4	80	20	linear
3.0	0.4	100	0	linear
7.0	0.4	100	0	linear
8.0	0.6	100	0	linear
11.6	0.6	100	0	linear
12.6[a]	0.4	80	20	linear

[a]After this time, the system was left for an additional 2 min to re-equilibrate before injection of a new sample.

Each dried sample was dissolved in 300 or 900 μL (for light and dark color extracts, respectively) of the injection solvent [ACN/MeOH 7:3, v/v]/acetone 6.7:3.3, v/v, for LC analysis. All solutions were filtered through Millex 0.2 μm nylon membrane syringe filters prior to introduction into the LC system (Millipore, Bedford, MA, USA).

MS analyses were conducted by atmospheric pressure chemical ionization (APCI), and the conditions used are the same as those described by Rivera et al.[21]

UV−Vis Spectroscopy. Absorption spectra and absorbance were recorded using a UV−vis spectrometer UV2 ATI UNICAM, Cambridge, UK.

Preparation of Carotenoid Standards. On the basis of the polarity of each carotenoid, ethanol was used to prepare stock solutions of violaxanthin and lutein; acetone for zeaxanthin; and hexane for β-cryptoxanthin, lycopene, β-carotene, and phytoene.[22,27] The concentration of these pigments was determined spectrophotometrically. For astaxanthin and canthaxanthin, a mixture of [ACN/MeOH 7:3, v/v]/acetone 6.7:3.3, v/v, was used to prepare stock solutions. In this case, concentrations were determined by dividing the mass of the carotenoid by the total volume of solution. Stock carotenoid solutions of violaxanthin, astaxanthin, canthaxanthin, zeaxanthin, lutein, β-cryptoxanthin, lycopene, β-carotene, and phytoene were prepared at concentrations of 16.26, 5.12, 5.70, 32.31, 21.57, 35.00, 8.26, 24.85, and 16.16 μg/mL, respectively. A set of standard solutions was prepared from stock solutions by sampling an aliquot and diluting it with the injection solvent, and their concentrations were assessed by UHPLC analysis. For those carotenoids dissolved in hexane (β-cryptoxanthin, β-carotene, lycopene, and phytoene), standard solutions were prepared from stock solutions by evaporating an aliquot under nitrogen and diluting it with the injection solvent. The $A^{1\%,\ 1cm}$ used to quantify each carotenoid can be found in Rivera et al.[22]

Identification and Quantification of Carotenoids. Carotenoids in samples were quantified using a PDA detector through the external standard method. Identification of carotenoids was carried out by analysis and comparison of the following parameters: chromatographic retention time (RT), UV−vis spectra, %III/II (this indicates the relationship between the peak heights of the longest wavelength absorption band, designated III, and that of the middle absorption band, designated II, taking the minimum between the two peaks as baseline, multiplied by 100),[27] and m/z fragments according to the authentic standards and literature data.[21] Those carotenoids for which there were no standards were assessed using the standard curves of the most similar carotenoids considering their structures and properties. Thus, the concentrations of adonixanthin and adonirubin were determined using the calibration curve of astaxanthin, those of 3-hydroxyechinenone and echinenone using the calibration curve of canthaxanthin, and that of β-zeacarotene using the calibration curve of β-carotene.

Table 2. Linear Regression Data, LOD, and LOQ Obtained with the UHPLC-PDA Technique[c]

carotenoid	linear range (μg/mL)	slope	intercept	LOD (μg/mL)	LOQ (μg/mL)	R^2
violaxanthin[a]	0.03−16.26	2516 ± 5.59	−121.93 ± 12.97	0.02	0.05	0.9994
astaxanthin[b]	0.04−5.12	1825 ± 6.43	−50.37 ± 10.51	0.02	0.06	0.9999
lutein[a]	0.02−17.25	2475 ± 81.74	−626.20 ± 35.78	0.05	0.14	0.9952
zeaxanthin[a]	0.03−17.23	2578 ± 38.04	−86.96 ± 25.8	0.03	0.10	0.9996
canthaxanthin[b]	0.02−5.70	1787 ± 4.24	−43.96 ± 16.13	0.03	0.09	0.9995
β-cryptoxanthin[a]	0.04−18.67	2379 ± 0.35	−444.17 ± 31.46	0.04	0.13	0.9988
lycopene[a]	0.3−3.11	1398 ± 104.40	−121.72 ± 24.88	0.06	0.18	0.9998
β-carotene[a]	0.1−24.85	1484 ± 27.22	−189.02 ± 29.80	0.07	0.20	0.9998
cis-phytoene[a]	0.08−16.16	1990 ± 285.46	−259.82 ± 37.32	0.06	0.19	0.9989

[a]Concentration was determined spectrophotometrically. [b]Concentration was determined by dividing the mass of the carotenoid by the total volume of solution. [c]Injection solvent: [ACN/MeOH 7:3, v/v]/acetone 6.7:3.3, v/v.

Table 3. Method Accuracy and Relative Recoveries in Maize Samples

							relative recovery (%)		
level	theor concn(μg/mL)	*N*	mean measd concn (μg/mL)	SD	%RSD	%Er	mean	SD	%RSD
β-Carotene									
low	0.48	6	0.46	0.03	6.13	−3.7	96	7.67	7.96
high	5.52	6	4.54	0.20	4.40	−18	82	4.34	5.27
Zeaxanthin									
low	0.35	5	0.36	0.02	5.52	3.9	104	8.15	7.83
high	3.93	5	3.66	0.19	5.19	−6.8	93	4.98	5.34
Astaxanthin									
low	0.14	5	0.14	0.01	9.88	2.1	102	10.40	10.17
high	5.66	5	5.55	0.29	5.18	−2.0	98	3.62	3.69
Lycopene									
low	0.69	5	0.43	0.06	13.15	−38	62	7.03	11.35
high	1.79	5	1.03	0.11	10.55	−42	58	4.97	8.56
Lutein									
low	0.55	6	0.54	0.03	5.29	−2.0	98	7.29	7.43
high	5.57	6	5.41	0.18	3.34	−2.8	97	4.81	4.94
β-Cryptoxanthin									
low	0.30	5	0.33	0.02	6.65	7.7	108	7.37	6.84
high	3.20	5	3.34	0.10	2.91	4.3	104	5.77	5.53

RESULTS AND DISCUSSION

Validation Method. Calibration curve estimations, LOD, LOQ, precision, and relative recovery were investigated to evaluate the analytical method. Each validation parameter is discussed in more detail in the subsequent paragraphs.

Calibration Curve Estimations. Calibration curves were obtained by injecting known concentrations of mixtures of standards and recording the resulting area. Between 5 and 10 standard solutions were prepared to determine the calibration curve, and three replicate measurements were made for each standard solution. Table 2 shows the calibration curve obtained for each carotenoid standard. In general, the carotenoid calibration curves exhibited a good linearity over the concentration range studied. Correlation coefficients (R^2) obtained were from 0.9999 to 0.9952 (Table 2).

LOD and LOQ. The LOD and LOQ were calculated using the equations

$$\mathrm{LOD} = 3.3\mathrm{Sb}/a$$

$$\mathrm{LOQ} = 10\mathrm{Sb}/a$$

where a is the curve slope and Sb is the standard deviation (SD) of the intercept.[28]

The LOD for carotenoids ranged from 0.02 to 0.07 μg/mL and the LOQ from 0.05 to 0.20 μg/mL (Table 2).

Relative Recovery, Precision, and Accuracy. The relative recovery of six standards was determined at two concentration levels (Table 3) by spiking the "blank" samples with the appropriate concentration and extracting according to the described method (see Materials and Methods). In addition, "blank" unspiked samples were extracted concurrently. The "blank" sample was the white maize variety M37W, which contains very few carotenoids at very low concentrations. The samples were analyzed after reconstitution in solvent. The relative recovery was determined by comparing the response ratios of samples from spiked maize to the response ratios of concentration of fortification.

$$\text{relative recovery} = (C_1 - C_2)/C_c \times 100\%$$

C_1 = concentration determined in spiked maize, C_2 = concentration determined in unfortified sample, and C_3 = concentration of fortification

Relative recoveries of β-carotene, zeaxanthin, lutein, astaxanthin, and β-cryptoxanthin ranged from 82 to 108% (Table 3) at the level tested. However, the relative recovery for lycopene ranged from 58 to 62%. Its low recovery may be attributed to the fact that lycopene is not as stable as other carotenoids,[29,30] and/or it may have a lower solubility in the solvents used during the extraction process. With the exception of lycopene, satisfactory recoveries were obtained for the other

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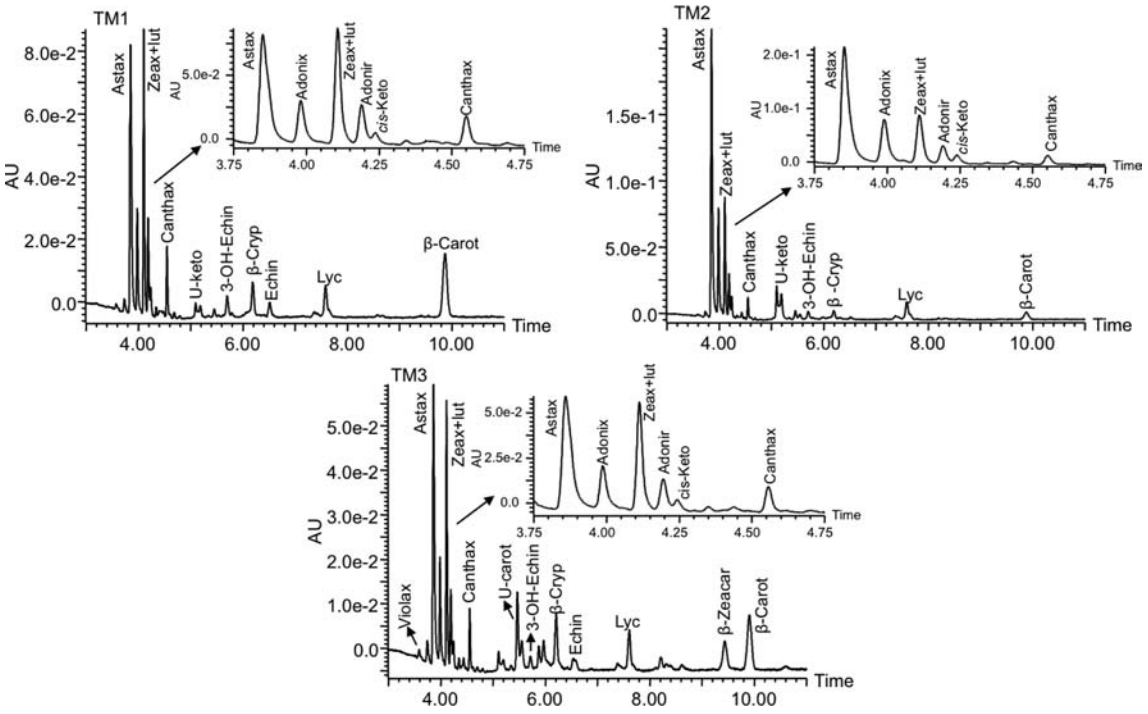

Figure 1. Carotenoid profile in TM1, TM2, and TM3. Abbreviations: Violax, violaxanthin; Astax, astaxanthin; Zeax, zeaxanthin; Lut, lutein; Adonix, adonixanthin; Adonir, adonirubin; *cis*-Keto, *cis*-unknown ketocarotenoid; U-keto, unknown ketocarotenoids; Canthax, canthaxanthin; U-cart, unknown carotenoid; 3-OH-Echinen, 3-hydroxyechinenone; Echin, echinenone; *β*-Cryp, *β*-cryptoxanthin; Lyc, lycopene; *β*-Zeacar, *β*-zeacarotene; *β*-Carot, *β*-carotene. The wavelength used for acquisition of chromatograms was 475 nm.

tested analytes within the indicated validation interval. Konings et al.[31] reported stock solutions of lutein, zeaxanthin, *β*-carotene, and lycopene with the same solvents used in this study. However, they used a mixture of MeOH/tetrahydrofuran (THF) 7.5:2.5, v/v, as injection solvent. Under those chromatographic conditions, they reported a higher linear range for lutein, zeaxanthin, and *β*-carotene than for lycopene. The smaller linearity range of lycopene (from 0 to 3.5 μg/mL) was explained by its lower solubility in the injection solvent. Nevertheless, the choice of the injection solvent was a compromise between satisfactory solubility of carotenoids, compatibility with the mobile phase, and the absence of peak distortions.

The accuracy was expressed as relative error (%Er) and determined as follows:[32,33]

$$\%\mathrm{Er} = [(\text{mean of measd concn} - \text{theor concn}) / \text{theor concn}] \times 100$$

The %Er obtained from the lowest concentration of *β*-carotene, zeaxanthin, lutein, astaxanthin, and *β*-cryptoxanthin was below 7.7% (Table 3), whereas from the highest concentration, it was below 15% (Table 3) except for *β*-carotene (%Er = 18). Considering that %Er values should be below 15%[33,34] for acceptance, a satisfactory level of accuracy was observed for most of the carotenoids within the studied concentrations. %Er of lycopene, up to 42%, was clearly outside the range of acceptance, which was due to its poor recovery, as discussed above.

The precision was estimated by the evaluation of the intraday precision (repeatability). The intraday precision was determined by calculating the relative standard deviation (%RSD) as

$$\%\mathrm{RSD} = (\mathrm{SD}/\bar{x}) \times 100\%$$

where SD is the sample standard deviation and $\bar{x}$ is the mean value of the sample data set.

The %RSD values for all analytes were below 13.15 and 10.55% for low and high concentration levels, respectively (Table 3). For acceptance, %RSD values should be below 15%.[33,34] Therefore, a satisfactory precision was observed within the studied concentrations.

In general, the results obtained showed that the method fulfills the performance characteristics demanded for the analysis of carotenoids in maize samples. This indicates adequate linearity, recovery, precision, accuracy, and sensitivity (LODs < 0.075 μg/mL).

Determination of Carotenoids in Maize Seeds. Once the reliability of the method was demonstrated, transgenic maize lines expressing several carotenogenic genes were analyzed. In these samples, a great diversity of carotenoids was found including ketocarotenoids (astaxanthin, adonixanthin, adonirubin, canthaxanthin, 3-hydroxyechinenone, and echinenone), an epoxycarotenoid (violaxanthin), carotenols (zeaxanthin, lutein, *α*-cryptoxanthin, and *β*-cryptoxanthin), and carotenes (lycopene, *β*-zeacarotene, *α*-carotene, *β*-carotene, and *cis*-phytoene). Using this method, effective baseline resolution was achieved for the xanthophylls and carotenes. Three representative chromatograms of the pigments detected in the transgenic maize lines TM1, TM2, and TM3 are shown in Figure 1. Table 4 shows the total and individual carotenoid contents found in these samples.

Preliminary Tests To Identify Carotenoids Present in the Transgenic Lines. Although most of the carotenoids present in the different TM were identified, some chromatographic peaks remained unidentified. In lines TM1 and TM2 there are three putative carotenoids with RT between 5.0 and 5.25 min. These unknown pigments are labeled in the

Table 4. Carotenoid Content and Composition in TM1, TM2, and TM3[a]

carotenoid	TM1 (μg/g DW)	TM2 (μg/g DW)	TM3 (μg/g DW)
zeaxanthin + lutein	3.52 ± 0.01	4.54 ± 0.32	4.43 ± 0.27
violaxanthin			0.24 ± 0.02
astaxanthin	7.07 ± 0.15	18.83 ± 0.57	10.87 ± 0.51
adonixanthin	1.93 ± 0.02	6.28 ± 0.33	3.11 ± 0.58
adonirubin	1.78 ± 0.08	2.33 ± 0.21	2.23 ± 0.08
cis-keto	0.58 ± 0.04	1.12 ± 0.02	0.83 ± 0.02
U-keto	0.82 ± 0.07	5.48 ± 0.01	
U-carot			3.23 ± 0.26
canthaxanthin	1.37 ± 0.05	1.15 ± 0.04	1.69 ± 0.05
3-OH-Echin	0.57 ± 0.03	0.64 ± 0.01	0.51 ± 0.00
β-Cryp	1.30 ± 0.08	0.86 ± 0.07	2.95 ± 0.10
echinenone	0.55 ± 0.01		0.88 ± 0.08
lycopene	2.41 ± 0.16	4.51 ± 0.26	4.13 ± 0.62
β-zeacarotene			3.69 ± 0.18
β-carotene	5.40 ± 0.31	2.02 ± 0.14	6.80 ± 0.61
cis-phytoene	0.73 ± 0.01	0.89 ± 0.04	10.02 ± 0.21
total concn	28.02 ± 0.81	48.63 ± 1.19	55.62 ± 3.57

[a]Abbreviations: *cis*-keto, unknown *cis*-ketocarotenoid; U-keto, unknown ketocarotenoids; U-carot, unknown carotenoid; 3-OH-Echin, 3-hydroxyechinenone; β-Cryp, β-cryptoxanthin; total concn, total carotenoid concentration.

chromatograms as U-keto (Figure 1). Figure 2A shows that these three pigments were not properly separated under the UHPLC conditions used. Hence, we modified the analysis parameters (e.g., gradient elution, flow rate, column temperature, etc.) to improve their resolution. Figure 2B shows the best separation obtained for these compounds and their UV–vis spectra. The carotenoid with RT at 2.81 min exhibited a λ_{max} at 468 nm and a symmetrical spectrum shape. Therefore, these spectral characteristics indicated that this compound should be a ketocarotenoid. Similarly, the spectral characteristics determined for the two unknown pigments coeluting at 3.01 min (Figure 2B) suggested that one or both of these pigments might be ketocarotenoids.

Mass spectra were also determined. Figure 2C shows the MS-APCI mass spectra for these molecules. Ions at m/z 567–568 and 549 have been already observed for 3-hydroxyechinenone and correspond to the $[M + H]^+$ and $[M + H - H_2O]^+$ ions, respectively.[21] Thus, these compounds might be 3′-hydroxyechinenone and geometrical isomers of 3′- or 3-hydroxyechinenone. Figure 3 shows the molecular structures of these carotenoids. This suggestion is based on these preliminary results and the analysis of the carotenoid biosynthetic pathway.

TM3 contains another unidentified compound, at 5.46 min. This compound is indicated in the chromatograms as "U-carot" (Figure 1). Although its UV–vis and mass spectra were determined (data not shown), it was not possible to suggest a structure because little information could be concluded from its mass spectrum. However, because its absorption appears between 400 and 500 nm and its protonated molecule seems

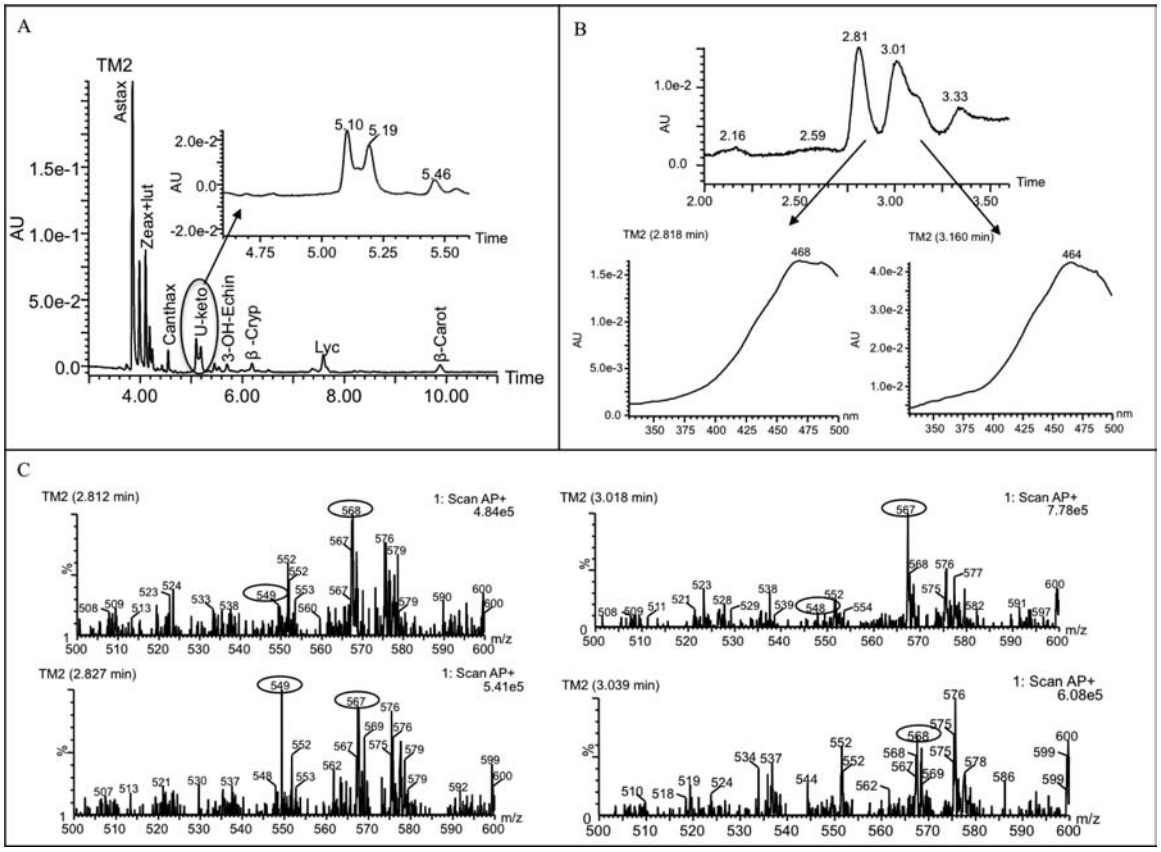

Figure 2. (A) Separation of the carotenoids present in TM1 and TM2; (B) UV–vis spectra of the unknown carotenoids found in TM2; (C) positive ion APCI mass spectra of the unknown pigments in TM2. The wavelengths used for acquisition of chromatograms in panels A and B were 475 and 465 nm, respectively.

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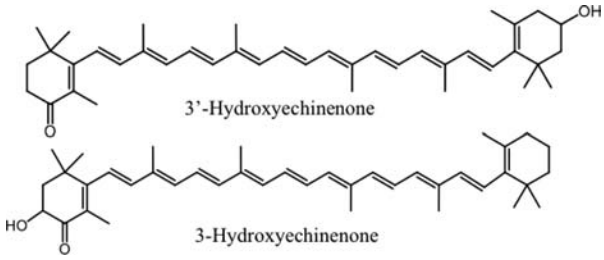

Figure 3. Structures of 3- and 3′-hydroxyechinenone.

to be at m/z 570, this unknown compound may be a carotenoid.

Appropriateness of the UHPLC System for the Profiling of Carotenoids. Table 5 shows the RT, UV−vis, and mass spectrometric characteristics of all carotenoids encountered in the maize samples in addition to the standards analyzed. Two transitions were used to identify each carotenoid: a quantifier (Q1) and a qualifier (Q2). These transitions were obtained by carrying out improvements on our previous work.[21] Despite the several modifications made to the UHPLC system to separate lutein and zeaxanthin, it was not possible to do so using an Acquity UPLC C18 BEH 130 Å, 1.7 μm, 2.1 × 100 mm column.

Investigations to improve the biosynthesis of carotenoids in transgenic maize require intensive data analysis because large and distinct sample populations are obtained. Consequently, it would be advantageous to reduce the chromatographic analysis time of these pigments without affecting the resolution or reliability of the analytical method. Thus far, a longer time is needed to determine the content of complex carotenoid mixtures such as those of ketocarotenoids or carotenoid geometric isomers. For example, the separation of astaxanthin, adonixanthin, adonirubin, lutein, canthanxanthin, 3-hydroxyechinenone, echinenone, and β-carotene required 45 min using an Ultrasphere C18, 250 × 4.6 mm, column. The mobile phase consisted of MeOH/dichloromethane (DCM)/ ACN/water 69:17:11.5:2.5, v/v.[35] Similarly, HPLC analysis of transgenic carrot leaves took around 45 min, allowing the separation of lutein, zeaxanthin, α-carotene, β-carotene, astaxanthin, adonixanthin, adonirubin, canthaxanthin, β-cryptoxanthin, and echinenone. These compounds were separated on a reverse phase YMC C30 carotenoid column using a mobile phase consisting of MeOH and *tert*-butyl methyl ether (MTBE) and a linear gradient between the two solvents.[36] The new method reported here allowed us to analyze 18 carotenoids including ketocarotenoids and geometric and positional isomers such as *cis*- and *trans*-phytoene and α- and β-carotene, respectively, in <13 min. Thus, this method has several advantages for carotenoid analysis, including reducing analysis time and solvent consumption. In addition, this method was shown to be reliable for the quantitative determination of several carotenoids in maize seeds.

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Notes

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Table 5. Chromatographic, UV−Vis, and Mass Spectrometric Characteristics of Carotenoids

carotenoid	RT[a] (min)	λ_{max}[b] (nm)	λ_{max} (nm) reported[c]	%III/II[b]	%III/II reported[c]	transition (m/z) Q1;[d] collision energy (eV)	transition (m/z) Q2;[d] collision energy (eV)
violaxanthin	3.58	417, 440, 470	419, 440, 470, ethanol	91	95	601.4 → 93; 45	601.4 → 133.3; 40
astaxanthin	3.85	476	478, ethanol			597.6 → 147; 40	597.6 → 579.6; 15
adonixanthin[e]	3.98	465	465, ethanol			583.4 → 147; 40	583.4 → 135.1; 40
zeaxanthin	4.11	453, 479	452, 479, acetone	25	25	569.4 → 135.1; 30	569.4 → 93; 40
lutein	4.11	446, 474	445, 474, ethanol	59	60	569.4 → 69; 40	569.4 → 135.1; 30
adonirubin[e]	4.19	475	474, acetone			581.5 → 147; 40	581.5 → 203.1; 40
canthaxanthin	4.53	472	474, ethanol			565.9 → 203.1; 40	565.9 → 69; 40
β-apo-8′-carotenal	4.77	459	456, ethanol			417.5 → 94.9; 25	417.5 → 325.3; 10
3-hydroxyechinenone[e]	5.68	464	466, ethanol			567.3 → 147; 40	567.3 → 93; 50
α-cryptoxanthin[e]	5.96	447, 475	446, 473, ethanol	64	60	553.6 → 461.6; 15	553.6 → 119; 35
β-cryptoxanthin	6.19	453, 479	450, 478, ethanol	23	25	553.6 → 119; 35	553.6 → 135.1; 30
echinenone[e]	6.45	461	461, ethanol			551.6 → 69; 45	551.6 → 93; 35
lycopene	7.58	446, 472[f]	446, 472, 504, ethanol		65	537.7 → 69; 40	537.7 → 93; 50
β-zeacarotene[e]	9.39	428, 454	428, 454, ethanol	40	52	539.6 → 69.3; 35	539.6 → 447; 10
α-carotene	9.46	447, 475	448, 476, acetone	50	55	537.6 → 123.1; 40	537.6 → 95.1; 35
β-carotene	9.74	453,478	452,478, acetone	13	15	537.6 → 68.9; 40	537.6 → 95.1; 35
cis-phytoene	10.98	286	286, hexane			545.5 → 81; 35	545.5 → 69; 35
trans-phytoene	11.31	286, 298	285, 297, hexane			545.5 → 81; 35	545.5 → 69; 35

[a]Retention time. [b]λ_{max} and %III/II obtained in the mobile phase, gradient elution of ACN/MeOH (7:3, v/v) and water. [c]λ_{max} and %III/II reported in the literature. [d]The most sensitive transition was used to generate a quantifier (Q1) and the second most sensitive transition to generate a qualifier (Q2). [e]Compounds not identified with standards. [f]The PDA detector used reads wavelengths up to only 500 nm; hence, the third maximum absorption of lycopene was not observed.

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