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Phenolic Compounds, Carotenoids, Anthocyanins, and Antioxidant Capacity of Colored Maize (*Zea mays* L.) Kernels

Slađana Žilić,[†] Arda Serpen,[§] Gül Akıllıoğlu,[∥] Vural Gökmen,*[,§] and Jelena Vančetović[‡]

[†]Department of Technology and [‡]Breeding Department, Maize Research Institute, Zemun Polje, Slobodana Bajića 1, RS-11085 Belgrade-Zemun, Serbia

[§]Food Research Center and [∥]Department of Food Engineering, Hacettepe University, 06800 Beytepe, Ankara, Turkey

ABSTRACT: In this study, the contents of total phenolics, flavonoids, anthocyanins, β-carotene, and lutein as well as free, conjugated, and insoluble bound phenolic acids were determined in whole kernels of 10 different colored maize genotypes. In addition, the antioxidant activity was evaluated as radical scavenging activity with ABTS (2,2-azino-bis/3-ethil-benothiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) reagents. Generally, considerable differences in phytochemical contents and antioxidant capacity were observed between the genotypes. The β-carotene and lutein contents ranged from 0 to 2.42 mg/kg d.m. and from 0 to 13.89 mg/kg d.m., respectively, whereas the total anthocyanin contents of anthocyanin-rich colored maize genotypes ranged from 2.50 to 696.07 mg CGE/kg d.m. (cyanidin 3-glucoside equivalent) with cyanidin 3-glucoside (Cy-3-Glu) as the most dominant form. The light blue ZPP-2 selfed maize genotype has a higher content of total phenolics, flavonoids, and ferulic acid as compared to other tested maize and the highest ABTS radical scavenging activity.

KEYWORDS: *colored maize, phenolics, carotenoids, anthocyanins, antioxidant capacity*

INTRODUCTION

Maize is one of the most diverse grain crops found in nature and one of the most widely cultivated cereals in the world. Also, maize and milled maize including meals, flours, and bran have been integral parts of the diet of all socioeconomic classes worldwide. Native white and pigmented maize have been cultivated in South America, mainly in Peru and Bolivia, and have been used for the preparation of traditional drinks and desserts long before European settlers arrived. However, multicolored, red, purple, blue, and black maize kernels are currently produced only in small amounts for making specialty foods or for use in ornamentation due to their colorful appearance.[1]

Pigmented maize contains many secondary metabolites, such as phenolic compounds and carotenoids. Phenolic acids and flavonoids represent the most common form of phenolic compounds found in whole maize kernel, with a number of types that exist as soluble free and conjugated or insoluble bound forms. More than 5000 flavonoids have been identified in nature.[2] The most significant function of the sap-soluble flavonoids is their ability to impart color to the plant in which they occur. Flavonoids are responsible for most orange, scarlet, crimson, mauve, violet, and blue colors, as well as contributing much to yellow, ivory, and cream colors.[3] Anthocyanins as a class of flavonoids are water-soluble glycosides of polyhydroxy and polymethoxy derivates of 2-phenylbenzopyrylium or flavylium salts and are responsible for the red, purple, and blue colors of many fruits, vegetables, and cereal kernels.[4] Simple or acylated anthocyanin pigments are mainly located in the aleurone layer or pericarp of the maize endosperm, greatly affecting the color of the kernel,[5] and could be separated into anthocyanin-rich fractions for use as functional colorants or functional food ingredients.[6] In addition to the color that they impart, there is an intensified interest in anthocyanins, as well as other flavonoids and phenolic acids, due to their beneficial health effects. The health beneficial properties of these plant metabolites have been attributed to their high antioxidant and antiradical activities but also to many other mechanisms such as antimutagenesis, anticarcinogenesis, and estrogenic activities, inhibition of enzymes, and induction of detoxification enzymes.[7] Anthocyanin-rich foods and anthocyanin pigments have been suggested as potential agents to reduce the risk of colon cancer by inhibiting proliferation of human colon cancer cells in vitro.[8] Also, the tests of Tsuda et al.[9] provide a nutritional and biochemical basis for the use of the anthocyanins as a "functional food factor" that may be beneficial for helping to prevent diabetes and obesity.

More than 600 carotenoid species have been identified in nature. Carotenoids are localized in subcellular organelles, chloroplasts, and chromoplasts, where thay are chiefly associated with proteins and serve as accessory pigments in photosynthesis. Two classes of carotenoid pigments, carotenes and xanthophylls, are responsible for the yellow and orange color of maize endosperm.[10] In general, α- and β-carotene are the major carotenes, whereas β-cryptoxanthin, lutein, and zeaxanthin make up the majority of the xanthophylls. In addition to the high antioxidant activities, α- and β-carotene and β-cryptoxanthin have provitamin A activities, while lutein and zeaxanthin have attracted much attention due to the possible role in preventing cataracts[11] and age-related macular degeneration.[12] Structurally, vitamin A (retinol) is essentially one-half of the β-carotene molecule.[13]

The present study was conducted to observe the levels of phenolic compounds, carotenoids, and antioxidant capacity of 10 different colored maize genotypes and to identify anthocyanin compositions using liquid chromatography−mass

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spectrometry (LC-MS) as a tool. Identifying maize genotypes with naturally rich pigments would promise a potential for the development of functional foods and/or functional food colorants.

MATERIALS AND METHODS

Chemical and Reagents. All chemicals and solvents were of high-performance liquid chromatography (HPLC) or analytical grade. Potassium persulfate (dipotassium peroxdisulfate) and cellulose were purchased from Fluka Chemie AG (Buchs, Switzerland). Methanol, acetone, sodium hydroxide, hydrochloric acid, formic acid, tetrahydrofuran, ethyl acetate, diethyl ether, ethanol, β-carotene, sodium carbonate, sodium nitrite, and aluminum chloride were purchased from Merck (Darmstadt, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin−Ciocalteu reagent, 2,2′-azino-bis/3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferulic acid, *o*- and *p*-coumaric acid, gallic acid, (+)-catechin, cyanidin 3-glucoside (kuromanin), pelargonidin 3-glucoside (callistephin), cyanidin 3,5-diglucoside (cyanin chloride), pelargonin 3,5-diglucoside, and lutein were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was used throughout the experiments (Milli-Q system, Millipore, Bedford, MA).

Plant Materials. A collection of 10 genotypes (landrace and inbred line, over the year 2010) of maize (*Zea mays* L.) was evaluated for antioxidants content. The genotypes were chosen on the basis of kernel color, as well as their differences in agronomic traits such as yield and its components. Their names, origins, and kernel types are given in Table 1. Maizes were grown in the field at the Maize Research Institute (Belgrade, Serbia), in a randomized complete block design (RCB) with two replications. Standard cropping practices were applied to provide adequate nutrition and to keep the disease-free plots. Maize samples were milled on a Perten 120 lab mill (Perten, Sweden) to a fine powder (particle size <500 μm). All whole maize flours were stored at −70 °C before analysis, and the analysis was performed within 2 months.

Table 1. Name, Origin, Genotype, and Kernel Type of the 10 Maize Genotypes[a]

genotype (name)	kernel type	genotype type	origin	country of origin
ZPL-1	semident	inbred line	CYMMIT (tropical)	Mexico
ZPL-2	semident-semiflint	inbred line	BSSS	United States
ZPL-3	dent	inbred line	Iowa dent-local population	United States
ZPL-4	flint	inbred line	F-2—local population Lancaune	France
ZPL-5	dent	inbred line	Lancaster + Iowa dent population	United States
ZPL-6	dent	inbred line	Lancaster-sure crop population	United States
ZPP-1 selfed	popcorn	landrace	MRI gene-bank	Serbia
ZPP-2 selfed	popcorn	landrace	MRI gene-bank	Serbia
ZPL-7	dent	inbred line	Iowa dent-local population	United States
ZPP-3	dent	landrace	local population	Nederland

[a]CYMMIT, International Maize and Wheat Improvement Centre; BSSS, Iowa Stiff Stalk Syntetic; and MRI, Maize Research Institute.

Extraction of Soluble Free, Soluble Conjugated, and Insoluble Bound Phenolic Compounds. The free phenolics, soluble conjugated, and insoluble bound phenolic acids in maize samples were extracted according to the procedure described by Moore et al.[14] The soluble free, soluble conjugated, and insoluble bound phenolic acids were extracted following a combined solvent and pH extraction and fractionation technique after alkaline-catalyzed release of bound phenolic acids from the solid maize matrix. A mixture of acetone/methanol/water (7:7:6, v/v/v) was used to extract the free and soluble conjugated phenolic acids. The insoluble phenolic acids in the residue and conjugated phenolic acids in the acetone/methanol/water extract were released by alkaline hydrolysis for 4 h at room temperature using 4 N NaOH before extraction. After the pH was adjusted to 2.0 by 6 N HCl, all of the hydrolysates were extracted with ethyl acetate and diethyl ether (1:1, v/v) four times. The combined extracts were evaporated under N_2 stream at 30 °C to dryness. The final residues were redissolved in 1.5 mL of methanol. After they were filtered through a 0.45 μm nylon filter, samples were kept at −20 °C prior to HPLC analysis.

Analysis of Individual Phenolic Acids. Chromatographic analyses were performed on an Agilent 1200 HPLC system consisting of a photodiode array detector, quaternary pump, autosampler, and column oven. Prior to injection, extracts were filtered through 0.45 μm nylon syringe filters (Phenomenex, Torrrance, CA). Phenolic acids were separated on a Waters Atlantis C18 column (250 mm × 4.6 mm, 5 μm) using a linear gradient elution program with a mobile phase containing solvent A (formic acid/H_2O, 1:99, v/v) and solvent B (pure methanol) at a flow rate of 0.8 mL/min with the following gradient profile: linear gradient elution from 10 to 60% B, 0−15 min; isocratic elution of 60% B, 15−20 min; linear gradient elution from 60 to 10% B, 20−25 min; and isocratic elution of 10% B, 25−30 min. The chromatograms were recorded at 280 nm by monitoring spectra within the wavelength range 190−400 nm. Identification of phenolic acids was accomplished by comparing the retention time and absorption spectra of peaks in maize samples to those of standard compounds. The quantitation of phenolic acids was based on calibration curves built for each of the compounds identified in the samples. The content of phenolic acids is expressed as μg per g of dry matter (d.m.).

Analysis of Anthocyanins. A ultrahigh performance chromatography (U-HPLC) was performed on a U-HPLC Accela system (Thermo Fisher Scientific, San Jose, CA) consisting of a degasser, a quaternary pump, an autosampler, and a column oven. Chromatographic separation was carried out on a Hypersil Gold AQ RP-C18 column (100 mm × 2.1 mm i.d., 1.9 μm). The mobile phase composed of a combination of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v) was used at a flow rate of 300 μL/min 30 °C. The linear gradient was from 10 to 30% B (v/v) at 10 min, to 100% B at 20 min, held at 100% B to 30 min, back to 10% B at 31 min, and held at 10% B to 35 min. The U-HPLC was directly interfaced to an Exactive Orbitrap mass spectrometer (MS) (Thermo Fisher Scientific). The Exactive Orbitrap MS equipped with a heated electrospray interface (HESI) was operated in the positive mode scanning the ions in m/z range of 100−1500. The resolving power was set to 50000 full width at half-maximum resulting in a scan time of 0.5 s. An automatic gain control target was set into high dynamic range, and the maximum injection time was 100 ms. The interface parameters were as follows: the spray voltage was 4.8 kV, the tube lens was at 70 V, the capillary voltage was 15 V, the capillary temperature was 350 °C, and a sheath and auxiliary gas flow of 40 and 15 arbitrary units were used, respectively. The instrument was externally calibrated by infusion of a calibration solution (m/z 138 to m/z 1822) by means of an automatic syringe injector (Chemyx Inc. Fusion 100T, United States). The calibration solution (Sigma-Aldrich) contained caffeine, Met-Arg-Phe-Ala, ultramark 1621, and acetic acid in the mixture of acetonitrile:methanol:water (2:1:1, v/v). Data were recorded using Xcalibur software version 2.1.0.1140 (Thermo Fisher Scientific).

Stock solutions of cyanidin 3-glucoside, pelargonidin 3-glucoside, cyanidin 3,5-diglucoside, and pelargonin 3,5-diglucoside were prepared in methanol acidified with 1 N HCl (85:15, v/v) at a concentration of 1.0 mg/mL. The working solutions were prepared by diluting the stock solutions with acidified methanol to concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, and 50.0 μg/mL. Identification of anthocyanins was accomplished by comparing the retention time and exact mass of peaks in the samples to those of standard compounds. The quantitation of individual anthocyanins was based on the calibration curves built for each compounds, and the results were expressed as mg per kg of d.m. Tentative identification of certain anthocyanins was also realized by comparing their theoretical masses with measured masses in high-resolution Orbitrap MS.

Analysis of Total Phenolic Content. The total phenolic content was determined according to the Folin−Ciocalteau procedure.[15] Briefly, the appropriate extracts (100 μL) were transferred into test tubes, and their volumes were made up to 500 μL with distilled water and oxidized with the addition of 250 μL of Folin−Ciocalteau reagent. Then, the mixture was neutralized with the addition of 1.25 mL of 20% aqueous Na_2CO_3 solution after 5 min of reaction. Mixtures were allowed to stand at ambient temperature for 40 min until the characteristic blue color developed; centrifugation was then carried out for 5 min at 4000*g*. Absorbance of the clear supernatants was measured at 725 nm against a blank containing an extraction solvent instead of sample. The total phenolic content of each sample was determined by means of a calibration curve prepared with gallic acid and expressed as mg of gallic acid equivalents (GAE) per kg of d.m.

Analysis of Total Flavonoid Content. The total flavonoid content was determined according to Zhishen et al.[16] Briefly, 50 μL of 5% $NaNO_2$ was mixed with 100 μL of the appropriate extracts. After 6 min, 500 μL of a 10% $AlCl_3$ solution was added to form a flavanoid−aluminum complex. After 7 min, 250 μL of 1 M NaOH was added, and the mixture was centrifuged at 5000*g* for 10 min. The absorbance of the supernatant was measured at 510 nm against the blank containing the extraction solvent instead of a sample. The total flavonoid content was expressed as mg of catechin equivalents (CE) per kg of d.m.

Analysis of Total Anthocyanin Content. Anthocyanins were extracted according to the method described by Abdel-Aal and Hucl[17] with slight modifications. Flour (500 mg) was extracted by mixing with 10 mL of methanol acidified with 1 N HCl (85:15, v/v) and shaking for 30 min at ambient temperature. The crude extract was centrifuged at 8000*g* for 20 min, and absorbances of supernatant at 535 and 700 nm were measured to detect anthocyanins. Anthocyanin levels were expressed as mg of cyanidin 3-glucoside equivalents (CGE) per kg of d.m., using the molar extinction coefficient of 25965 Abs/M × cm and a molecular weight of 449.2 g/mol.

Analysis of Carotenoids. Carotenoids were extracted and analyzed according to a method described by Hentschel et al.[18] with some minor modifications. Briefly, 200 mg of whole maize flour was mixed with 50 mg of Na_2CO_3 and extracted with 1.5 mL of methanol/tetrahydrofuran (1:1, v/v) solution for 10 min. Extraction was done by repeated stirring four times for 10 min at ambient temperature, followed by centrifugation at 7500*g* for 5 min. The combined extracts were evaporated to dryness under nitrogen gas at 35 °C and redissolved in 1 mL of methanol/tetrahydrofuran (1:1, v/v). The carotenoid extracts were filtered through a nylon syringe filter (0.45 μm) (Phenomenex) and analyzed in a Agilent 1200 HPLC system equipped with a photodiode array detector and a C18 column (Agilent Zorbax, 250 mm × 4.6 mm, 3.5 μm) using solvent A (methanol), solvent B (water), and solvent C (tetrahydrofuran) as the mobile phase. The solvent gradient was programmed as described by Serpen et al.[19] Identification of lutein and β-carotene was accomplished by comparing the retention time and absorption spectra of peaks in maize samples to that of standard lutein and β-carotene, and then, the quantitative data were calculated from their linear calibration curves under analysis conditions. The results for the lutein and β-carotene were expressed as mg per kg d.m.

Analysis of Total Antioxidant Capacity. Measuring of the total antioxidant capacity was done based on QUENCHER method described by Serpen et al.[20] using ABTS, as well as DPPH reagents. A stock solution of $ABTS^{\bullet+}$ was prepared by mixing a 7 mM aqueous solution of $ABTS^{\bullet+}$ with 2.45 mM $K_2O_8S_2$ (final concentration) and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. On the day of analysis, an $ABTS^{\bullet+}$ working solution was obtained by diluting the stock solution in water/ethanol (50:50, v/v) to overcome the solubility-dependent low reactivity of antioxidants in solid sample toward $ABTS^{\bullet+}$. The absorbance of the $ABTS^{\bullet+}$ working solution was 0.70 ± 0.02 AU at 734 nm. A working solution of DPPH reagent was prepared in 50% ethanol with final concentration of 1 mM. The absorbance of the $DPPH^{\bullet}$ working solution at 525 nm was 1.0 ± 0.05 AU. Maize flour (10 mg) was mixed by adding 20 mL of $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ working solutions, and the mixture was rigorously shaken for 25 min. After centrifugation at 9200*g* for 5 min, the optically clear supernatant was separated, and the absorbance measurement was performed at 734 and 525 nm, respectively. The antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (TEAC) in mmol of Trolox per kg of d.m.

Statistical Analysis. The analytical data were reported as means ± standard deviations of least duplicate independent extractions. Significant differences between genotype means were assessed by the Fisher's least significant differences (LSD) test, after the analysis of variance (ANOVA) for trials set up according to the RCB design. Differences with $p < 0.05$ were considered significant. Correlations between parameters were examined using the Pearson's correlation test.

RESULTS AND DISCUSSION

The pro-vitamin A carotenoid, the β-carotene, was identified in the seventh of maize genotypes analyzed (Table 2). The highest content of β-carotene was found in orange maize ZPL-4 (2.42 mg/kg d.m.), while genotypes with lemon yellow, dark red, and light blue color of kernels were found to be free of β-carotene. In the white (ZPL-1), yellow (ZPL-3), and red (ZPL-6) genotypes, the amount of β-carotene was lower than 0.75 mg/kg d.m. It should be noted that the maize kernel color, among other things, strictly depends on conjugated double bonds and the various functional groups contained in the carotenoid molecule.[13] Naturally occurring β-carotene, with 11 double bonds, is orange in color.[21] The red (ZPL-6)- and orange (ZPL-4)-colored genotypes showed greater potential as sources of carotenoids due to their high concentration of lutein (13.89 and 11.14 mg/kg d.m., respectively). In contrast, a very low concentration of lutein (<0.08 mg/kg d.m.) was detected for the lemon yellow-, dark red-, and light blue-colored genotypes ZPL-2, ZPL-1 selfed, and ZPL-2 selfed (Table 2). As lutein can absorb blue light, it appears as yellow color.[22] Kurilich and Juvik[23] reported that lutein accounted for 57% of the carotenoids in 44 maize cultivars, while zeaxanthin and β-cryptoxanthin made up 21 and 5% of the carotenoids, respectively. Only 8% of the total carotenoids were carotenes. Our results are well in accordance with data reported for golden yellow maize[24] and different colored waxy maize genotypes[25] but higher than that of Brazilian colored maize.[26] Given the potential of some of the analyzed genotypes, their use in official or local breeding programs aimed at increasing the content of carotenoids of new maize genotypes, as well as in preparation of functional foods, is thought to be relevant. This fact is extremely important given that the vitamin A deficiency may cause acquired blindness in children and nonocular systemic consequences including increased infectious morbidity and mortality, growth retardation, and anemia.[27] In Africa, where white maize is the most important staple food, devastating effects of vitamin A deficiency are attributed to over 4−6% of the entire disease burden.[28]

Coloration of maize kernel is derived also from the accumulation of anthocyanins. White maize had a lower total flavonoid content (248.64 mg CE/kg d.m.) than those of red and dark red ones (267.58 and 270.54 mg CE/kg d.m., respectively) as well as light and dark blue maize (337.51 and 307.42 mg CE/kg d.m., respectively) (Table 2). It should be noted that red and dark red maize were lower in flavonoid contents than lemon yellow, yellow, and orange maize. It was possible that other flavonoid compounds (e.g., flavonols and flavones) rather than anthocyanins in the yellow and orange maize were higher than those in the red and blue maize genotypes. The results are comparative to those found for rice.[29] It is possible that in addition to carotenoids, flavonols and flavones contributed to cream and yellow colors of maize ZPL-1, ZPL-2, ZPL-3, and ZPL-4 in which the total flavonoid contents

Table 2. Content of Natural Pigments and Flavonoids in Different Colored Maize Kernels[a]

Genotype (name)	ZPL-1	ZPL-2	ZPL-3	ZPL-4	ZPL-5
Kernel colour	white	lemon yellow	yellow	orange	red-yellow
					
Lutein[1]	n.d.	0.08±0.00^{g}	5.91±0.31^{d}	11.14±0.62^{b}	9.59±0.30^{c}
β-carotene[1]	0.21±0.08^{f}	n.d.	0.70±0.14^{e}	2.42±0.30^{a}	1.15±0.06^{d}
Flavonoids[2]	248.64±3.92^{e}	280.42±15.20^{cd}	281.20±2.18^{cd}	293.59±2.18^{bc}	268.35±6.52^{de}
Anthocyanins[3]	n.d.	n.d.	n.d.	n.d	2.50±0.06^{f}

Genotype (name)	ZPL-6	ZPP-1 selfed	ZPP-2 selfed	ZPL-7	ZPP-3
Kernel colour	red	dark red	light blue	dark blue	multicolored
					
Lutein[1]	13.89±0.67^{a}	0.01±0.00^{g}	0.01±0.00^{g}	5.34±0.52^{e}	4.63±0.15^{f}
β-carotene[1]	0.60±0.13^{e}	n.d.	n.d.	1.68±1.86^{b}	1.40±0.28^{c}
Flavonoids[2]	267.58±3.49^{de}	270.54±3.26^{cde}	337.51±13.04^{a}	307.42±17.42^{b}	198.99±13.03^{f}
Anthocyanins[3]	15.43±1.64^{e}	696.07±2.73^{a}	378.92±4.89^{c}	597.15±6.54^{b}	139.12±1.63^{d}
Cy-3-Glu[4]	0.07±0.01^{d}	547.49±8.80^{a}	308.24±5.64^{b}	312.43±6.98^{b}	54.71±1.64^{c}
Cy-3,5-diGlu[4]	n.d.	1.12±0.13^{c}	1.65±0.09^{a}	1.36±0.10^{b}	0.08±0.01^{d}
Pg-3-Glu[4]	n.d.	5.12±0.16^{a}	1.87±0.08^{b}	1.34±0.06^{c}	0.41±0.03^{d}
Pg-3,5-diGlu[4]	n.d.	n.d.	1.65±0.19^{a}	n.d.	n.d.

[a]Key: [1]mg/kg d.m. [2]mg CE/kg d.m. [3]mg CGE/kg d.m. [4]mg/kg d.m. Means of genotypes followed by the same letter within both rows of the same characteristics are not significantly different ($p > 0.05$); n.d., not detected.

Table 3. High-Resolution Mass Spectral Identification of Certain Anthocyanins in Colored Maize Kernels[a]

		m/z+			
athocyanin	molecular formula	theoretical	experimental	mass accuracy (ppm)	identified maize kernel
Cy-3-Glu	$C_{21}H_{21}O_{11}$	449.10784	449.10797	0.29	ZPL-6, ZPP-1 selfed, ZPP-2 selfed, ZPL-7, ZPP-3
Cy-3,5-diGlu	$C_{27}H_{31}O_{16}$	611.16066	611.16083	0.28	ZPP-1 selfed, ZPP-2 selfed, ZPL-7, ZPP-3
Pg-3-Glu	$C_{21}H_{21}O_{10}$	433.11292	433.11313	0.48	ZPP-1 selfed, ZPP-2 selfed, ZPL-7, ZPP-3
Pg-3,5-diGlu	$C_{27}H_{31}O_{15}$	595.16575	595.16620	0.76	ZPP-2 selfed
Cy-3,6-MalGlu	$C_{24}H_{23}O_{14}$	535.10823	535.10840	0.32	ZPL-6, ZPP-1 selfed, ZPP-2 selfed, ZPL-7, ZPP-3
Pn-3-Glu	$C_{22}H_{23}O_{11}$	463.12349	463.12378	0.63	ZPP-1 selfed, ZPL-7, ZPP-3
Pn-3,6-MalGlu	$C_{25}H_{25}O_{14}$	549.12388	549.12408	0.36	ZPP-1 selfed, ZPP-2 selfed, ZPL-7, ZPP-3
Pg-3,6-MalGlu	$C_{24}H_{23}O_{13}$	519.11332	519.11377	0.87	ZPP-1 selfed, ZPP-2 selfed, ZPL-7, ZPP-3
De-3-Glu/De-3-Gal	$C_{21}H_{21}O_{12}$	465.10275	465.10248	−0.58	ZPP-1 selfed, ZPP-2 selfed, ZPL-7, ZPP-3
Cy-3-Rut	$C_{27}H_{31}O_{15}$	595.16575	595.16608	0.55	ZPP-1 selfed, ZPP-2 selfed, ZPL-7, ZPP-3
Cy-3,6-EthylMalGlu	$C_{26}H_{27}O_{14}$	563.13953	563.13977	0.43	ZPP-1 selfed, ZPP-2 selfed, ZPL-7
Pn-3,6-EthylMalGlu	$C_{27}H_{29}O_{14}$	577.15518	577.15503	−0.26	ZPL-6, ZPP-1 selfed, ZPP-3
Pg-3,6-EthylMalGlu	$C_{26}H_{27}O_{13}$	547.14462	547.14498	0.66	ZPL-6, ZPP-1 selfed

[a]Cy, cyanidin; Glu, glucoside; Mal, malonyl; Pg, pelargonidin; Pn, peonidin; De, delphinidin; Gal, galactoside; Et, ethyl; and Rut, rutinoside.

were increased with the intensity of the kernel's yellow color. Several reports have shown that cyanidin, pelargonidin, and peonidin glycosides are the main anthocyanins present in maize kernels.[1,30,31] Blue, pink, purple, red, and multicolored maize exhibit complex anthocyanin composition having 18−27 compounds.[1] Cyanidin derivatives accounted around 70% of the anthocyanins.[31] According to our results, the maize kernels having red and blue colors were found to contain a wide concentration range of total anthocyanins from 2.50 to 696.07 mg CGE/kg d.m. Dark red maize (ZPP-1 selfed) had the highest concentration of total anthocyanins followed by dark blue (ZPL-7), light blue (ZPP-2 selfed), and multicolored (ZPP-3) maize (Table 2). Cyanidin 3-glucoside (Cy-3-Glu) was found as the most dominant form of anthocyanins in these maize kernels. Pelargonidin 3-glucoside (Pg-3-Glu), cyanidin 3,5-diglucoside (Cy-3,5-diGlu), and pelargonin 3,5-diglucoside (Pg-3,5-diGlu) were detected only in trace amounts in red- and blue-colored maize kernels (Table 2). High-resolution orbitrap mass spectrometry analyses allowed identification of several anthocyanins tentatively in colored maize kernels. As given in Table 3, a total of 13 anthocyanins could be detected with high mass accuracy (<1.0 ppm), confirming their presence in certain samples. In the kernels of 15 Mexican black, purple, red, and blue pigmented maize genotypes, anthocyanin contents ranged from 76.2 to 869 mg CGE/100 g d.m.[32] However, our results are well in accordance with data reported by Abdel Aal et al.[1] for different colored maize. In contrast to results of Lopez-Martinez et al.,[32] as well as Mora-Rochin et al.,[33] anthocyanins have not been detected in white, yellow, and orange maize kernels, and this was expected as these colorations would indicate that

carotenoids, particularly in the form of lutein and zeaxanthin, are the most abundant pigment types in light colored maize.[23] Our results are in accordance with literature data for Mexican white maize.[34] Generally, the anthocyanins with many OH groups in a molecule give a blue color, whereas the presence of many OCH_3 groups shifts the color toward red.[35] A great deal of research has been conducted on the anthocyanins in fruits and vegetables. However, given the anthocyanins health benefits as dietary antioxidants, identification of low-cost anthocyanin-rich sources with increased stability and their practical applications in food industries are very important. In addition, the anthocyanin pigments in maize kernels can be concentrated by dry milling and fractionation processes to produce fractions that are high in anthocyanin contents, even much higher than those found in fruits and vegetables.[1]

Red and blue maize are especially high in phenolic compounds as compared to light colored maize genotypes.[25,34] Total phenolic contents of tested maize genotypes are presented in Table 4. In the maize genotypes free of anthocyanin, (ZPL-1, ZPL-2, ZPL-3, and ZPL-4), the content of total phenolics ranged from 5227.1 to 5811.2 mg GAE/kg d.m. having no significant difference between lemon yellow, yellow, and orange genotypes ($p > 0.05$). Among the tested anthocyanin-rich genotypes, the highest total phenolic content was detected in light blue ZPP-2 selfed maize followed by dark blue ZPL-7 maize having the value of 10528.8 and 7352.5 mg GAE/kg d.m., respectively. No significant differences were found between different red genotypes (ZPL-5, ZPL-6, and ZPP-1 selfed) ($p > 0.05$). The average value of these three genotypes for total phenolics was 6056.9 mg GAE/kg d.m., which was about 57% from that determined in light blue maize. The multicoloured maize had the lowest content of total phenolics (4494.1 mg GAE/kg d.m.). The obtained range of tested maize samples was similar as compared with the total phenolic content values of 15 yellow, red, purple, and black maize genotypes grown in Mexico[32] but higher than those reported for U.S.-grown white, yellow, red, and blue maize, which was in the range of 243.8−285.8 mg GAE/100 g d.m.[10]

Three individual phenolic acids were detected in colored maize genotypes, namely, ferulic, *p*-coumaric, and *o*-coumaric acids. The contents of soluble free, soluble conjugated, insoluble bound, as well as the total phenolic acids calculated as the sum of these fractions, are given in Table 4. As shown in percentage contribution of phenolic acid fractions, the bound forms are the major phenolic acid forms in maize genotypes. Results show that approximately 76−91% of the total ferulic and *p*-coumaric acid in maize kernels is in the bound form. However, it was interesting that a low content of *o*-coumaric acid was determined in free and esterified forms contributing from 51.65 to 100% and from 0 to 48.35% to the total phenolic acid, respectively, whereas the insoluble bound fraction of *o*-coumaric acid was not found. As the predominant phenolic acid in maize kernel, ferulic acid is mainly associated with arabinoxylans and other polysaccharides present within cell walls of the aleuronic layer.[36] Because of its semielastic properties, this compound has an ability to form complexes with pentosans and proteins and is important in the formation of the dough texture.[37] Among tested maize genotypes, the light blue ZPP-2 selfed genotype contained the highest total ferulic acid content (4521.26 μg/g d.m.) followed by lemon yellow ZPL-2 and dark blue ZPL-7 maize. The content of total ferulic acid in other tested genotypes ranged from 1556.24 to 2859.81 μg/g d.m. Our results for total ferulic acid are much higher than those reported by Lopez-Martinez et al.[32] for different colored maize genotypes but lower than that reported by Mora-Rochin et al.[33] for Mexican white, yellow, red, and blue maize.

The QUENCHER method with ABTS, as well as DPPH reagents, was used to determine the antioxidant capacity of 10 maize genotypes, and these values were compared to the amounts of total phenolics, flavonoids, anthocyanins, cyanidin 3-glucoside, phenolic acids, and carotenoids determined in each of the genotypes (Figure 1 and Table 5). It is expected that a different order of antioxidant capacity of different colored maize was obtained by different methods (Figure 1). The direct procedure that skips all time-consuming solvent extraction and hydrolysis steps was applied, and it can be concluded that the antioxidant capacity of maize kernel antioxidant system depends upon which free radical is used in the assay.[38] These results confirm previous research[19] that $ABTS^{\bullet+}$ is more sensitive to phenolic-containing compounds than $DPPH^{\bullet}$. ZPP-2 selfed, ZPL-7, and ZPP-1 selfed maize genotypes have light blue, dark blue, and dark red kernel colors, respectively, containing the highest levels of total phenolics, anthocyanins, and antioxidant capacity of the genotypes tested. According to the obtained data, it could be said that a higher content of phenolic compounds in the maize kernel contributes to their higher antioxidant activity. These results are in well accordance with literature data.[25] Also, from our data, it can be concluded that a darker colored maize has more antioxidant capacity. Lopez-Martinez et al.[32] also found that pigmented black, purple, red, and blue maize showed relatively higher radical scavenging activity than nonpigmented samples. The differences in the antioxidant capacity among red, blue, and purple colored maize genotypes are possibly related to the specific composition of anthocyanin derivatives such as simple or acylated glycosides of cyanidin, pelargonidin, or peonidin.[39] The radical scavenging capacity of different anthocyanins depends mainly on the number of hydroxyl groups and their position on the molecule. Additionally, glycosylation of the anthocyanidins may modulate the antioxidant capacity depending on the aglycons.[40] No significant positive correlation was found between ABTS radical scavenging activities and lutein and β-carotene contents. In the genotypes with no anthocyanin pigment accumulation that shows various kernel colors ranging from white to orange, the intensity of maize kernel coloration was closely related to antioxidant capacity. The genotypes had ABTS radical scavenging activities with the following descending order: orange > yellow > lemon yellow > white (ZPL-4 > ZPL-3 > ZPL-2 > ZPL-1). It can be assumed that the antioxidant capacity of studied carotenoids was disguised with strong antioxidant capacity of anthocyanins in the samples of different red and blue maize. However, significant positive correlation coefficients between β-carotene and antioxidant capacity ($r^2 = 0.70$) and lutein and antioxidant capacity ($r^2 = 0.87$) determined only among the no anthocyanin maize confirm that the carotenoids influenced the capacity of $ABTS^{\bullet+}$ scavenging. Terao[41] reported that the presence of nine or more double bonds and oxo groups at the 4(4′)-position in the β-ionone ring in the carotenoid structure greatly enhanced the singlet oxygen quenching activity. Significant correlations between insoluble bound ferulic acid contents and ABTS radical scavenging activities point to their contribution to antioxidant capacity. The antioxidant capacity was positively correlated with the portion of pericarp per kernel weight (Table 5). Kernels of small to medium size produce darker red and blue flour coloration and a higher concentration of phenolic compounds per g of d.m. because they possess a higher proportion of aleurone and bran layer and are less diluted by the

Table 4. Content of Total Phenolics (mg GAE/kg d.m.) and Individual Phenolic Acids (μg/g d.m.) in Different Colored Maize[a]

		ZPL-1	ZPL-2	ZPL-3	ZPL-4	ZPL-5	ZPL-6	ZPP-1 selfed	ZPP-2 selfed	ZPL-7	ZPP-3
total phenolics		5227.1 ± 331.2 e	5778.2 ± 36.7 cd	5393.2 ± 10.1 de	5811.2 ± 143.1 cd	6011.4 ± 227.9 c	6044.5 ± 204.3 c	6114.7 ± 162.2 c	10528.8 ± 58.8 a	7352.5 ± 498.5 b	4494.1 ± 293.8 f
phenolic acids*											
ferulic	free soluble	3.36 ± 0.58 g (0.12)	4.63 ± 0.42 f (0.14)	8.75 ± 0.21 b (0.31)	10.59 ± 0.42 a (0.37)	5.46 ± 0.74 e (0.35)	3.45 ± 0.14 g (0.13)	4.70 ± 0.42 f (0.17)	7.60 ± 0.85 c (0.17)	5.92 ± 0.71 de (0.20)	6.18 ± 0.28 d (0.24)
	soluble conjugated	373.19 ± 7.52 e (13.43)	53.09 ± 15.71 b (13.83)	321.63 ± 7.72 g (11.25)	483.94 ± 12.74 a (16.69)	361.84 ± 8.39 f (23.25)	311.90 ± 2.69 h (11.50)	408.37 ± 12.17 d (14.14)	439.55 ± 5.57 c (9.72)	309.51 ± 8.41 h (10.27)	376.15 ± 10.61 e (14.54)
	insoluble bound	2401.42 ± 42.42 e (86.45)	2816.92 ± 21.03 b (86.02)	2529.42 ± 27.41 d (88.45)	2404.57 ± 12.34 e (82.94)	1188.93 ± 19.61 g (76.40)	2397.22 ± 13.38 e (88.37)	2422.50 ± 31.85 e (85.43)	4074.10 ± 39.94 a (90.11)	2697.03 ± 33.73 c (89.53)	2203.88 ± 5.55 f (85.22)
	total	2777.97 ± 50.52 f	3274.65 ± 37.16 b	2859.81 ± 35.40 d	2899.11 ± 5.50 d	1556.24 ± 28.74 i	2712.58 ± 16.22 g	2835.57 ± 44.45 e	4521.26 ± 56.36 a	3012.46 ± 42.85 c	2586.21 ± 16.45 h
p-coumaric	free soluble	1.25 ± 0.14 g (0.47)	2.73 ± 0.42 e (1.03)	5.94 ± 0.56 b (1.29)	9.89 ± 0.37 a (1.76)	4.04 ± 0.06 d (1.00)	2.85 ± 0.28 e (0.89)	1.84 ± 0.27 f (0.78)	2.76 ± 0.25 e (0.83)	5.12 ± 0.43 c (1.06)	2.84 ± 0.11 e (1.53)
	soluble conjugated	21.65 ± 0.91 g (8.18)	32.06 ± 3.10 e (12.09)	44.16 ± 3.09 c (9.58)	119.33 ± 5.32 a (21.28)	40.86 ± 2.37 d (10.11)	23.07 ± 2.16 f (7.21)	29.31 ± 2.35 e (12.39)	59.16 ± 3.83 b (17.75)	39.90 ± 1.74 d (8.24)	30.05 ± 2.76 e (16.13)
	insoluble bound	241.65 ± 10.62 f (91.35)	230.47 ± 6.06 f (86.88)	410.97 ± 17.88 b (89.13)	431.55 ± 5.18 a (76.96)	359.29 ± 14.57 c (88.89)	293.98 ± 7.91 d (91.90)	205.43 ± 2.95 g (86.83)	271.36 ± 5.22 e (81.42)	439.22 ± 14.45 a (90.70)	153.36 ± 3.35 h (82.34)
	total	264.55 ± 11.68 e	265.26 ± 9.59 e	461.09 ± 21.53 b	560.77 ± 10.87 a	404.19 ± 16.99 c	319.90 ± 10.35 d	236.58 ± 5.57 f	333.29 ± 9.31 d	484.24 ± 16.63 b	186.25 ± 6.22 g
o-coumaric	free soluble	12.52 ± 0.14 g (56.59)	18.40 ± 0.42 d (75.72)	16.31 ± 0.45 f (100.00)	28.34 ± 0.70 a (71.31)	22.30 ± 0.42 b (68.09)	15.93 ± 0.47 f (69.58)	0.53 ± 0.01 h (63.74)	17.78 ± 0.39 e (65.63)	20.40 ± 0.56 c (51.65)	15.91 ± 0.43 f (47.24)
	soluble conjugated	9.61 ± 0.52 d (43.41)	5.90 ± 0.22 e (24.28)	ND	11.40 ± 0.90 c (28.69)	10.45 ± 0.82 cd (31.91)	6.97 ± 0.21 e (30.42)	0.30 ± 0.01 f (36.26)	9.31 ± 0.78 d (34.37)	19.10 ± 1.35 a (48.35)	17.77 ± 0.89 b (52.76)
	insoluble bound	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	total	22.13 ± 0.38 e	24.30 ± 0.65 d	16.32 ± 0.44 f	39.74 ± 1.61 a	32.75 ± 1.25 b	22.90 ± 0.68 de	0.83 ± 0.03 g	27.09 ± 1.17 c	39.50 ± 1.92 a	33.68 ± 1.31 b

[a]Means of genotypes followed by the same letter within the same row are not significantly different ($p > 0.05$); ND, not detected; *values in parentheses represent the percentage of different fractions contribution of phenolic acids to the total content.

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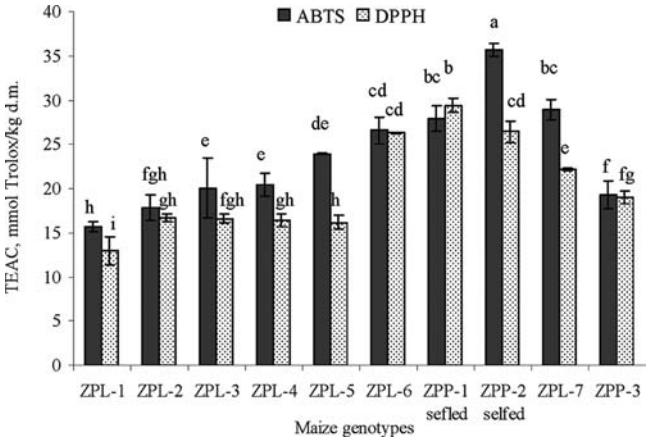

Figure 1. Total antioxidant capacity of different colored maize genotypes obtained by QUENCHER method with ABTS (■) and DPPH (□) reagents. The vertical bars represent the standard deviation of each data point. Bars with different letters are statistically significantly different ($p < 0.05$).

Table 5. Linear Correlations between the Content of Phytochemicals and Antioxidant Capacity[a]

	pericarp	TEAC (ABTS)	TEAC (DPPH)	total phenolics
TAC (ABTS)	0.72*			
TAC (DPPH)	0.60	0.80**		
total phenolics	0.87**	0.86**	0.52	
total flavonoids	0.59	0.47	0.01	0.77**
total anthocyanins	0.45	0.64*	0.72*	0.46
cyanidin 3-glucoside	0.48	0.67*	0.77*	0.50
lutein	−0.48	0.03	−0.05	−0.41
β-carotene	−0.58	−0.10	−0.30	−0.25
ferulic (soluble free)	−0.001	0.18	−0.17	0.18
ferulic (soluble conjugated)	0.34	−0.06	−0.08	0.19
Ferulic (insoluble bound)	0.55	0.69*	0.60	0.65*
p-coumaric (soluble free)	−0.35	0.01	−0.29	−0.06
p-coumaric (soluble conjugated)	0.03	0.14	−0.16	0.20
p-coumaric (insoluble bound)	−0.15	0.42	0.09	0.23
o-coumaric (soluble free)	−0.28	−0.08	−0.50	0.09
o-coumaric (soluble conjugated)	−0.23	−0.01	−0.18	0.06

[a] *, significant at $p < 0.05$; **, significant at $p < 0.01$.

starchy endosperm.[5] In our study, the light blue maize (ZPP-2 selfed) with the highest portion of pericarp per kernel weight (11.49%) (the results are not shown) had the highest content of total plenolics, flavonoids, ferulic acid, and ABTS radical scavenging activity (35.66 mmol Trolox/kg d.m.).

Our results provided the evidence of high levels of health beneficial phytochemicals and antioxidant capacity of colored maize. Significant differences were found in lutein and *β*-carotene contents for the 10 different colored maize genotypes. It can be concluded that the orange (ZPL-4) genotype has a superior potential for use as a source of pro-vitamin A compounds. The results of total anthocyanin contents indicate that some of the colored maize kernels such as dark red, dark blue, and light blue maize may hold promise for the development of functional foods and/or natural colorants.

AUTHOR INFORMATION

Corresponding Author

*Tel: +312 297 71 08. Fax: +312 299 21 23. E-mail: vgokmen@hacettepe.edu.tr.

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ABBERVATIONS USED

ABTS, 2,2-azino-bis/3-ethyl-benothiazoline-6-sulfonic acid; ANOVA, analysis of variance; CE, catechin equivalents; CGE, cyanidin 3-glucoside equivalents; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GAE, gallic acid equivalents; HPLC, high-performance liquid chromatography; LSD, least significant differences; RCB, randomized complete block design; TEAC, Trolox equivalent antioxidant capacity

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