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Soluble and Bound Phenolic Compounds in Different Bolivian Purple Corn (*Zea mays* L.) Cultivars

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ABSTRACT: In nine Bolivian purple corn (*Zea mays* L.) varieties the content of phenolic compounds as well as the anthocyanin composition has been determined. The phenotypes under investigation included four red and five blue varieties (Kulli, Ayzuma, Paru, Tuimuru, Oke, Huaca Songo, Colorado, Huillcaparu, and Checchi). In purple corn, phenolic compounds were highly concentrated in cell walls. Thus, simultaneous determination of soluble and bound-form phenolics is essential for analysis, extraction, and quantification. The present study reports the determination of soluble and insoluble-bound fraction of phenolic compounds by HPLC-DAD and HPLC-ESI-MS^n in Bolivian purple corn varieties. Enzymatic, thermal, and alkaline hydrolyses were used to obtain the cell wall-linked phenolic compounds. Ferulic acid values ranged from 132.9 to 298.4 mg/100 g, and *p*-coumaric acid contents varied between 251.8 and 607.5 mg/100 g dry weight (DW), respectively, and were identified as the main nonanthocyanin phenolics. The total content of phenolic compounds ranged from 311.0 to 817.6 mg gallic acid equivalents (GAE)/100 g DW, and the percentage contribution of bound to total phenolics varied from 62.1 to 86.6%. The total monomeric anthocyanin content ranged from 1.9 to 71.7 mg cyanidin-3-glucoside equivalents/100 g DW. Anthocyanin profiles are almost the same among the different samples. Differences are observed only in the relative percentage of each anthocyanin. Cyanidin-3-glucoside and its malonated derivative were detected as major anthocyanins. Several dimalonylated monoglucosides of cyanidin, peonidin, and pelargonidin were present as minor constituents.

KEYWORDS: *Zea mays* L., soluble phenolics, bound phenolics, *p*-coumaric acid content, ferulic acid content, anthocyanin content

INTRODUCTION

Purple corn (*Zea mays* L.) is a rich source of anthocyanins. For centuries, purple corn has been cultivated in South America, mainly in Peru and Bolivia, and used for the preparation of traditional drinks and desserts. Figure 1 shows different varieties of pigmented Bolivian corn. Pigmented corn contains many secondary metabolites, such as carotenoids and phenolic compounds.[1] These constituents are regarded as the most important source of antioxidants in cereals and exist in free as well as bound form. The majority of free phenolics are flavanols, whereas the bound phenolics are mainly phenolic acids. Nevertheless, the free forms of phenolic compounds are less abundant in comparison to their esters, glycosides, and bound complexes. For this reason, several hydrolytic procedures have been described to quantify the amount of total phenolics. Most of these procedures are based on enzymatic or alkaline hydrolysis with NaOH. Different phenotypes of maize have been shown to exhibit variations in their antioxidant activities and phenolic profiles. Several studies have reported a range of bioactivities of maize components, which included inter alia the antioxidant and anticarcinogenic effect of white corn constituents such as ferulic and *p*-coumaric acid along with their respective derivatives.[2]

Whereas *p*-coumaric acid is known to be mainly bound to lignin[3] with only smaller amounts attached to polysaccharides,[4,5] ferulic acid is mostly ester-linked to cell wall polysaccharides such as arabinoxylans or pectins.[6] Ferulates can also dehydrodimerize via a radical, oxidative mechanism to form mainly 8,5′-, 8-O-4′-, 5-5′-, 8-8′-, and 4-O-5′-coupled dehydrodiferulates (often simply referred to as diferulates) and oligomers,[7,8] thus cross-linking plant cell wall polymers and polysaccharide with important implications for physical and textural attributes of plants, as well as for human nutrition.[9,2] Purple-, black-, and red-pigmented maizes are rich in anthocyanins, which exhibit important antioxidant and bioactive properties. Although considered to be nonnutritive, anthocyanins from purple corn have been associated with multiple health benefits.[10] They possess high antioxidant activities,[11] reduce systolic blood pressure of hypertensive rats,[12] and prevent obesity and diabetes in mice.[13] Purple corn anthocyanins were found to reduce cell mutation induced by 2-amino-1-methyl-6-phenylimidazopyridine (PhIP)[14] and chemically induced colorectal carcinogenesis in male rats.[15] In purple corn, cyanidin-3-glucoside, cyanidin-3-(6″-malonylglucoside), and cyanidin-3-(3″,6″-dimalonylglucoside) have been determined as the major anthocyanins, with cyanidin being the main aglycone moiety (73−87% of the total).[16] Small amounts of pelargonidin derivatives and peonidin derivatives have been also reported to occur in the plant.[17,18] In this study, a method for preparative isolation of pure anthocyanins from purple corn was established using high-speed countercurrent chromatography (HSCCC). HSCCC is a modern and automated liquid−liquid chromatographic technique that has been used for the preparative isolation of numerous natural products because of the gentle isolation conditions and large yields of pure compounds.[19−21] Previous studies on pigmented maize varieties mainly focused on anthocyanin composition, and only some studies reported the

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Figure 1. Varieties of pigmented Bolivian purple corn cultivars.

phenolic content and antioxidant activity of purple corn.[11,22,23] Obviously, without a proper determination of the bound phenolic fraction linked to the cell wall materials, their phenolic content has most likely been underestimated. Therefore, more analyses of soluble and bound-form phenolics and anthocyanins of purple corn varieties are required. For this reason, the objective of this study was to determine the phenolic profiles, including both soluble and bound phenolic compounds, and anthocyanins of samples from nine pigmented corn varieties grown in Bolivia and to develop appropriate conditions for their separation and identification. To our knowledge, this is the first application of HSCCC for the preparative separation of Bolivian purple corn pigments on a large scale. Moreover, more insight was required with regard to cell wall bound phenolics in purple corn.

MATERIALS AND METHODS

Plant Material. The seeds of purple corn varieties Kulli, Ayzuma, Paru, Tuimuru, Oke, Huaca Songo, Colorado, Huillcaparu, and Checchi were generously supplied by Dr. Gonzalo Ávila from Centro de Investigaciones Fitoecogenéticas de Pairumani (CIFP), Fundación Patiño, Cochabamba, Bolivia. Prior to analysis, whole grain samples were ground to a fine powder by using an IKA laboratory mill (Janke & Kunkel Co., Staufen, Germany) and stored at −20 °C until extraction.

Solvents and Reagents. All reagents and solvents employed were of HPLC purity or analytical grade. Methanol and *tert*-butyl methyl ether (TBME) were redistilled prior to use. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Munich, Germany), deuterated NMR solvents were from Deutero (Kastellaun, Germany), and formic acid p.A., >98%, was from Acros Organics (Belgium). All enzymes (Amylase BAN 480 L, Cellulase Cellubrix L, and Pectinase Pectinex UF) were from Novozymes (Denmark). Water was demineralized by a Nanopure (Barnstead, IA) apparatus.

Isolation of Pigments by HSCCC. Pigment extraction was carried out according to the method previously described by Eichhorn and Winterhalter[24] and Hillebrand et al.[20] Approximately 450 g of purple corn flour was blanched at 100 °C for 3 min with 900 mL of water. The same volume of a solution of water/hydrochloric acid (19:1 v/v) was added. The suspension was stirred at 0 °C for 3 h and then stored at room temperature for 8 h without stirring. To remove solid material, the suspension was filtered prior to application onto an Amberlite XAD-7 column (Fluka, 100 × 7 cm), which had been conditioned with 2 L of methanol and then with 2 L of water. The column was rinsed with 3 L of water to remove sugars, proteins, organic acids, and minerals. The anthocyanins were eluted with 2 L of a mixture of methanol/glacial acetic acid (19:1 v/v). The eluate was concentrated in vacuo, dissolved in water, and freeze-dried. Between 2.1 and 2.7 g of purified XAD-7 extracts was obtained from 450 g of each of the samples of purple corn flour.

Subsequent isolation of the pigments was achieved by HSCCC. Separations were carried out with a high-speed model CCC-1000 (Triplecoil; diameter of tubing = 2.6 mm, total volume = 850 mL, revolution speed = 850 rpm) produced by Pharma-Tech Research Corp. (Baltimore, MD). A two-phase solvent system, consisting of TBME/*n*-butanol/acetonitrile/water (2:2:1:5 v/v/v/v, acidified with 0.1% TFA) was used (less dense layer as stationary phase) with a flow rate of 4 mL/min. An amount of 500 mg of each XAD-7 extract (redissolved in 22 mL of solvent mixture) was injected for a single run, and all CCC fractionations were monitored at 520 nm.

Extraction of Soluble Phenolic Compounds (E1). An amount of 8 g of purple corn flour was blended by Ultra Turrax apparatus with 40 mL of ethanol/water (80:20 v/v) for 2 min. After the flour was separated from the extract by filtration through a folded filter into a volumetric flask, the residue was washed with a 20 mL portion of formic acid (5%), and the final volume was adjusted to 100 mL by adding formic acid (5%).

Extraction of Bound Phenolic Compounds by Enzymatic Hydrolysis (E2). Bound phenolics were extracted from the residue of the soluble phenolic determination. The residue was suspended in 20 mL of $Ca(OH)_2$ (1 mM), the pH value was adjusted to 5.0, and the enzyme preparation (500 μL of pectinase, 500 μL of amylase, and 250 μL of cellulase) was added. The suspension was then stirred for 24 h at room temperature and filtered into a 100 mL flask. The residue was washed again with water, and the volume was adjusted to 100 mL.

Extraction of Bound Phenolic Compounds by Thermal Hydrolysis (E3). For thermal liberation of bound phenolic compounds, the residue from the enzymatic hydrolysis was first dried for 4 h in a vacuum at 70 °C and then dissolved in 50 mL of methanol. After boiling under reflux for 1 h, the resulting suspension was filtered into a 50 mL flask. The final volume was adjusted by adding methanol.

Extraction of Bound Phenolic Compounds Using Alkaline Hydrolysis (E4). The residue was washed with 5 mL of acetone and dried for 4 h in a vacuum at 70 °C. The dry residue was resuspended in 15 mL of 4 M NaOH and stirred for 2 h. The resulting slurry was acidified with 10 mL of concentrated HCl, and the emulsion was filtered into a 50 mL flask. The final volume was adjusted to 50 mL by water.

High-Performance Liquid Chromatography (HPLC). HPLC analyses were performed on an MD-910 multiwavelength detector (wavelength range between 220 and 650 nm), equipped with a DG-980-50 3-line degasser and an LG-980-02 ternary gradient unit, a PU-980 Intelligent HPLC pump, an AS-950 Intelligent autosampler, and Borwin PDA chromatography software (Jasco, Gross-Umstadt, Germany). HPLC separation was carried out on a Luna RP-18 column (250 × 4.6 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.5 mL/min, using an injection volume of 20 μL (in solvent system A). The binary gradient consisted of solvent system A (water/acetonitrile/formic acid 87:3:10 v/v/v) and solvent system B (water/acetonitrile/formic acid 40:50:10 v/v/v). Conditions were as follows: 0 min, 6% B; 20 min, 20% B; 35 min, 40% B; 40 min, 60% B; 45 min, 90% B; and 55 min, 6% B, followed by a 5 min equilibration period.

High-Performance Liquid Chromatography−Electrospray Ionization Multiple Mass Spectrometry (HPLC-ESI-MS^n). Freeze-dried samples (XAD-7 extracts as well as CCC fractions containing a mixture of anthocyanins) were redissolved in a mixture of

Table 1. Total Contents of Soluble and Bound Phenolics in Bolivian Purple Corn Cultivars Determined by Using the Folin−Ciocalteu Assay[a]

	phenolics (mg gallic acid equiv/100 g DW)				
	soluble	bound			
cultivar	E1	E2	E3	E4	total
Ayzuma	90.7	168.4	96.6	112.7	468.4
Paru	73.9	115.8	88.4	117.0	395.1
Tuimuru	102.3	123.1	77.1	128.2	430.7
Huaca Songo	72.6	109.6	52.4	129.3	363.9
Colorado	68.1	155.7	23.1	64.1	311.0
Huillcaparu	102.0	137.1	84.4	115.3	438.8
Checchi	77.1	119.7	37.3	151.7	385.8
Oke	86.5	136.9	66.6	124.3	414.3
Kulli	82.1	205.0	135.4	189.2	611.7
Kulli flour	309.7	178.3	159.1	170.5	817.6

[a] E1−E4, extraction methods (cf. Materials and Methods).

water/acetonitrile/formic acid 95:3:2 (v/v/v) and were analyzed by HPLC-ESI-MS^n. HPLC analyses were performed using an Agilent system (Böblingen, Germany) equipped with a binary pump (1100 series) and an autosampler (1200 series). The same analytical conditions as described above were used. ESI-MS^n measurements were performed on a Bruker Esquire-LC multiple ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass spectral analyses were recorded under the following operating conditions: positive and negative ion mode; capillary, −2500 V; capillary exit offset, 70 V; end plate offset, −500 V; skimmer 1, 20 V; skimmer 2, 10 V; dry gas, N_2, 11 L/min; dry temperature, 325 °C; nebulizer, 60 psi; scan range, m/z 50−2200. Esquire NT 4.0 software (Bruker Daltonics) was used for analysis and data collection.

Electrospray Ionization Multiple Mass Spectrometry (ESI-MS^n). For ESI-MS^n experiments pure compounds were redissolved in a mixture of water/acetonitrile/formic acid 95:3:2 (v/v/v) and were delivered directly via a syringe pump 74900 (Cole-Parmer, USA) into the ESI source (flow rate = 240 μL/h). Mass spectrometry parameters were the following: positive ion mode; capillary, −3500 V; capillary exit offset, 60 V; end plate offset, −500 V; skimmer 1, 30 V; skimmer 2, 10 V; dry gas, N_2, 4 L/min; dry temperature, 300 °C; nebulizer, 10 psi; scan range, m/z 50−1000.

Quantification of Total Phenolics, Anthocyanins, and *p*-Coumaric and Ferulic Acid. Total phenolic content in the extracts was determined using the Folin−Ciocalteu method,[25] with gallic acid as standard. Total phenolic values were reported in milligrams of gallic acid equivalents per 100 g of dry weight (mg GAE/100 g DW). For quantification of monomeric anthocyanins, *trans*-*p*-coumaric acid and *trans*-ferulic acid by HPLC analyses, five-point calibration curves of cyanidin-3-glucoside (3.2−50.8 mg/L), *trans*-*p*-coumaric and *trans*-ferulic acid (50−150 mg/L) were used. All anthocyanins were detected at 520 nm and were calculated as cyanidin-3-glucoside equivalents, whereas *p*-coumaric acid and ferulic acid were quantitatively determined at 320 nm. Values are the mean ± SD of three independent experiments.

RESULTS AND DISCUSSION

Total Phenolic Content. The soluble and bound phenolic contents of nine different purple corn cultivars are presented in Table 1 and are expressed as milligrams of gallic acid equivalents per 100 g of sample based on the dry weight (DW). The soluble phenolic content ranged from 68.1 to 309.7 mg GAE/100 g DW.

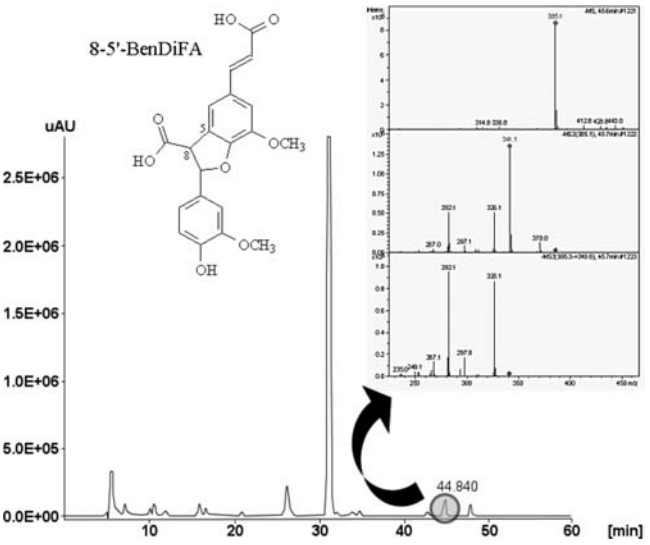

Figure 2. HPLC chromatogram of the bound phenolic extract of the variety Oke after alkaline hydrolysis at 320 nm, including mass spectrometric data and structure of the tentatively characterized *trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran 3-carboxylic acid (8-5′-BenDiFA).

For determination of bound-form phenolics, enzymatic cleavage with various enzyme preparations (pectinases, cellulases, amylases) has been reported to release phenolic acids.[26] Between 109.6 and 205.0 mg GAE/100 g DW was released by enzyme treatment (E2). Subsequent thermal treatment (E3) showed that by using enzymes, not all of the phenolic constituents could be cleaved from the cell walls. Between 23.1 and 159.1 mg GAE/100 g DW was released after thermal treatment (1 h of reflux at 70 °C). Eventually, hydrolysis under strong alkaline conditions (E4) released additional phenolic constituents in the range from 64.1 to 189.2 mg GAE/100 g DW. The total content of bound-form phenolics detected in Bolivian purple corn cultivars varied from 242.9 to 529.6 mg GAE/100 g DW. The sum of soluble and bound phenolics was in the range of 311.0−817.6 mg GAE/100 g DW. In our study, the highest amounts of phenolics were detected in the dark-blue variety Kulli (flour). The percentage contribution of bound to total phenolics ranged from 62.1 to 86.6%, indicating that the bound phenolics are the major phenolic fraction in purple corn varieties.

Determination of Phenolic Acid Content and Composition. Identification of monomeric soluble and bound-form phenolic compounds of the purple corn varieties was achieved by HPLC-DAD analyses using external standards. Two major hydroxycinnamic acid monomers were detected in all of the purple corn cultivars at retention times of 30.7 and 36.2 min, respectively. These compounds were identified as *p*-coumaric acid and ferulic acid. In addition to the monomers, further *p*-coumaric acid and ferulic acid derivatives were detected in bound-form fractions showing retention times between 40 and 50 min. By comparison with literature data,[27] these peaks were characterized as ferulic acid dehydrodimers and dehydrotrimers. One cyclic dimeric ferulic acid derivative with a retention time of 44.8 min was tentatively identified by ESI-MS^n spectroscopy as *trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran 3-carboxylic acid or 8-5′-Ben DiFA previously described by Boutigny et al.[28] in wheat bran. 8-5′-BenDiFA showed a pseudomolecular ion $[M - H]^-$ at m/z 385

Table 2. Contents of *p*-Coumaric Acid and Ferulic Acid in Bolivian Purple Corn Cultivars Determined by Using HPLC-DAD Analysis[a]

	phenolic acids (mg/100 g DW)							
	p-coumaric acid				ferulic acid			
	soluble	bound			soluble	bound		
cultivar	E1	E2	E3	E4	E1	E2	E3	E4
Ayzuma	19.3	54.9	13.3	279.2	99.8	11.4	21.7	nd
Paru	nd	16.2	5.7	292.4	135.5	nd	84.3	nd
Tuimuru	nd	64.9	16.4	336.3	148.5	27.8	48.5	nd
Huaca Songo	17.5	12.3	7.0	308.2	98.0	nd	92.5	nd
Colorado	nd	20.0	11.2	274.2	139.0	nd	159.4	nd
Huillcaparu	nd	41.3	17.3	193.2	126.7	nd	123.3	nd
Checchi	17.4	40.3	7.5	318.2	147.7	26.2	24.6	nd
Oke	20.0	45.7	11.3	331.7	175.3	18.0	22.2	nd
Kulli	34.1	107.0	27.0	439.4	135.2	nd	19.0	nd
Kulli flour	53.8	75.2	26.1	415.9	127.9	nd	14.0	nd

[a] E1−E4, extraction methods (cf. Materials and Methods). nd, not detected.

and fragments at *m*/*z* 341, 326, 297, 282, and 267.[28] Figure 2 shows the HPLC chromatogram of the bound phenolic extract of the variety Oke after alkaline hydrolysis including the mass spectrometric data and the structure of the 8-5′-benzofuran dimer of ferulic acid.

The quantification of free *p*-coumaric acid and ferulic acid showed that ferulic acid was the most dominant phenolic acid in all purple corn cultivars. Ferulic acid content ranged from 98.0 to 175.3 mg/100 g DW, whereas *p*-coumaric acid was found in moderate levels up to 53.8 mg/100 g DW for the cultivar Kulli (flour). The quantitative determination after enzymatic treatment (E2) showed that *p*-coumaric acid was the major phenolic acid in all cultivars (12.3−107.0 mg/100 DW). In four samples small amounts of ferulic acid could be detected by HPLC-DAD analysis. Up to 27.8 mg/100 g DW was determined in the cultivar Tuimuru. Most enzymes for analytical use contain no cinnamoyl esterase activity, and release of phenolic compounds is caused by cleavage of the ether, acetal, or hemiacetal linkage between the carbohydrate unit and the hydroxyl groups of the aromatic system.[18] For cleavage of ester-bonded phenolic acids two different methods have been published in the literature, the so-called acified hydrolysis and saponification by treatment with NaOH.[27,29] Small amounts of *p*-coumaric acid (up to 27.0 mg/100 g DW) and ferulic acid (up to 159.4 mg/100 g DW) were isolated by treating phenolics ester-linked to cell wall polysaccharides such as arabinoxylans and pectins with methanol under reflux conditions (E3).[27] In all samples *p*-coumaric acid bound to lignin is the major monomeric hydroxycinnamic acid. Amounts released by alkaline hydrolysis were in the range of 193.2−439.4 mg/100 g DW (E4).[27] Table 2 summarizes the contents of free and bound *p*-coumaric and ferulic acid. Our results show that the majority of phenolic acids are linked to cell wall polymers and are present in bound form, whereas only small amounts exist as soluble phenolic acids. By calculation of the total amount of major phenolic acids in the investigated Bolivian cultivars the values ranged from 251.8 to 607.5 mg/100 g DW for *p*-coumaric acid and from 132.9 to 298.4 mg/100 g DW for ferulic acid, respectively.

Table 3. Total Contents of Soluble and Bound Monomeric Anthocyanins in Bolivian Purple Corn Cultivars Determined by Using HPLC-DAD Analysis[s]

	anthocyanins (mg cy-3-glc equiv/100 g DW)				
	soluble	bound			
cultivar	E1	E2	E3	E4	total
Ayzuma	10.56	5.34	1.31	nd	17.21
Paru	nd	nd	nd	nd	nd
Tuimuru	27.64	11.91	11.48	nd	51.03
Huaca Songo	1.97	nd	nd	nd	1.97
Colorado	nd	nd	nd	nd	nd
Huillcaparu	nd	nd	nd	nd	nd
Checchi	7.53	nd	0.87	nd	8.40
Oke	14.71	3.54	5.16	nd	23.41
Kulli	51.25	15.07	3.02	2.34	71.68
Kulli flour	63.73	4.47	1.91	1.41	71.52

[s] E1−E4, extraction methods (cf. Materials and Methods). nd, not detected.

Determination of Anthocyanin Content and Composition. The monomeric anthocyanin content varied significantly between the varieties and is expressed as milligrams of cyanidin-3-glucoside equivalents (cy-3-glc equiv) per 100 g of DW. In the case of Paru, Colorado, and Huillcaparu no significant amounts of monomeric anthocyanins could be detected by HPLC-DAD analysis. The soluble content of anthocyanins ranged from 1.97 to 63.73 mg cy-3-glc equiv/100 g DW. The percentage contributions of bound anthocyanins to the total anthocyanins were in all samples below 50%. The contents of soluble and bound anthocyanins are summarized in Table 3. The varieties Kulli and Tuimuru had the highest concentrations of soluble and bound anthocyanins. These results demonstrated that some Bolivian cultivars of purple corn showed a high content of phenolic compounds and anthocyanins, thus constituting good sources for functional food development as well as natural food colorants.

The anthocyanin composition of Bolivian purple corn is mainly composed of non- and monoacylated derivatives.[16−18] There are numerous reports on the anthocyanin composition in various parts of the plant. A summary can be found in Mazza and Miniati[30] and Aoki et al.[31] The HPLC-DAD (520 nm) chromatogram of anthocyanins extracted from the Kulli cultivar is shown in Figure 3. Anthocyanin profiles are almost the same among the nine samples (data not shown). Differences are observed only in the relative percentage of each anthocyanin. With the HPLC conditions used in this study, at least 10 peaks were separated from the anthocyanin extracts of the nine genotypes analyzed. Ayzuma, Huaca Songo, Kulli, and Kulli flour varieties contained 10 anthocyanin pigments, the major peaks (24.5−35.9% peak area) consisting of cyanidin-3-glucoside (**2**) eluted at 17.6 min and cyanidin-3-(6″-malonylglucoside) (**5**) at 25.4 min. The varieties Tuimuru, Checchi, and Oke showed three major and seven minor pigments by HPLC analysis. Peaks 2, 5, and 7 are the predominant compounds (10.9−30.1% peak area) and were characterized as cyanidin-3-glucoside (**2**), cyanidin-3-(6″-malonylglucoside) (**5**), and peonidin-3-(6″-malonylglucoside) (**7**), respectively. Dimalonylated monoglucosides of cyanidin, peonidin, and pelargonidin could also be detected as minor pigments. Peaks 9 and 10 were tentatively identified as pelargonidin-3-(dimalonylglucoside) (**9**)

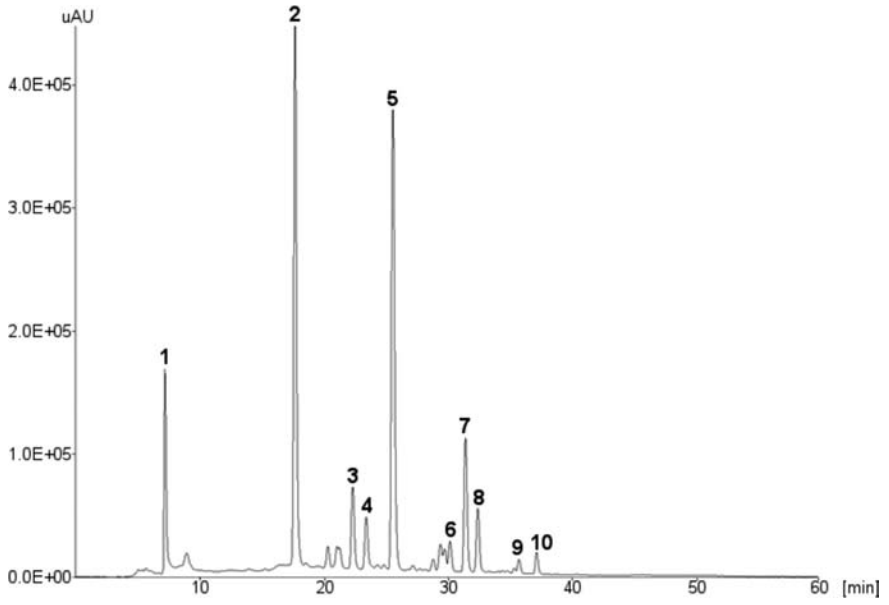

Figure 3. HPLC chromatogram of the anthocyanin-enriched XAD-7 extract of Bolivian purple corn Kulli at 520 nm. For compound labeling cf. Table 4.

Table 4. Mass Spectrometric Data and Identification of Anthocyanin Compounds Characterized from Kulli Corn Cultivar

			m/z			
peak[a]	retention time (min)	peak area[b] (%)	M^+	fragments	compound identity	ref
1	8.1	7.8	899	737, 575, 557, 449, 287	(epi)catechin−cyanidin-3,5-diglucoside	32
2	17.6	37.7	449	287	cyanidin-3-glucoside	17, 31
3	22.2	5.4	433	271	pelargonidin-3-glucoside	17, 31
4	23.3	3.8	463	301	peonidin-3-glucoside	17, 31
5	25.4	26.8	535	449, 287	cyanidin-3-(6″-malonylglucoside)	17, 31
6	30.1	1.7	519	433, 271	pelargonidin-3-(6″-malonylglucoside)	17, 31
7	31.3	7.5	549	463, 301	peonidin-3-(6″-malonylglucoside)	17, 31
8	32.3	3.6	621	535, 449, 287	cyanidin-3-(dimalonylglucoside)	10, 31
9	35.7	0.7	605	519, 433, 271	pelargonidin-3-(dimalonylglucoside)	*c*
10	37.1	1.1	635	549, 463, 301	peonidin-3-(dimalonylglucoside)	*c*

[a] Peaks are shown in Figure 3. [b] Relative area. [c] Tentatively identified in this work (cf. text).

and peonidin-3-(dimalonylglucoside) (**10**), respectively. In Table 4, the relative anthocyanin composition of the Kulli extract calculated from the peak areas obtained at 520 nm is summarized together with HPLC-ESI-MS^n data.

The identification of individual anthocyanin compounds is based on the data from HPLC-DAD/-ESI-MS^n analysis and by comparison with literature data.[16−18,31] In the following, an example shows HSCCC separation of the dark corn variety Kulli (Figure 4). HSCCC separated five fractions as well as the coil residue. The first fraction consisted of (epi)catechin−cyanidin-3,5-diglucoside (**1**). Mass spectrometric analysis of **1** showed a pseudomolecular ion $[M]^+$ at *m/z* 899 and fragments at *m/z* 737, 575 (successive loss of two hexose residues), 557 (loss of water), 449 (126 amu, loss of $C_6H_6O_3$), and 287 (288 amu, loss of an upper (epi)catechin unit).[32] The CCC fraction 2 (34 mg) contains pure cyanidin-3-glucoside (**2**) in a purity of 92%, which is the major pigment not only of the cultivar Kulli but also of other maize morado cobs.[1] CCC fraction 4 (36 mg) contains cyanidin-3-(6″-malonylglucoside) (**5**) in a purity of 85%. The structure of **5** was confirmed by HPLC-ESI-MS^n measurements. The pseudomolecular ion $[M]^+$ with *m/z* 535 yielded fragments of *m/z* 449 and 287 (Table 4). Fraction 3 consisted of a mixture of pelargonidin-3-glucoside (46%) (**3**) and peonidin-3-glucoside (**4**) (40%), which could be isolated in pure form by preparative HPLC.

In our studies, a method has been developed for the simultaneous determination of soluble and bound-form phenolics in nine Bolivian purple corn varieties. HPLC-DAD and HPLC-ESI-MS^n analyses of free and bound-form fractions demonstrated a wide variation in the phenolic contents and anthocyanin composition.

The percentage contribution of bound to total phenolics in the samples examined indicated that the bound phenolics are the major phenolic fraction in purple corn varieties, significantly higher than that of free and esterified phenolics. The present study shows that nonanthocyanin phenolic compounds should be taken into consideration when purple corn bioactive properties are studied. The knowledge generated from this study may help to exploit the use of purple corn as a functional ingredient and to promote its use in disease risk reduction and overall health.

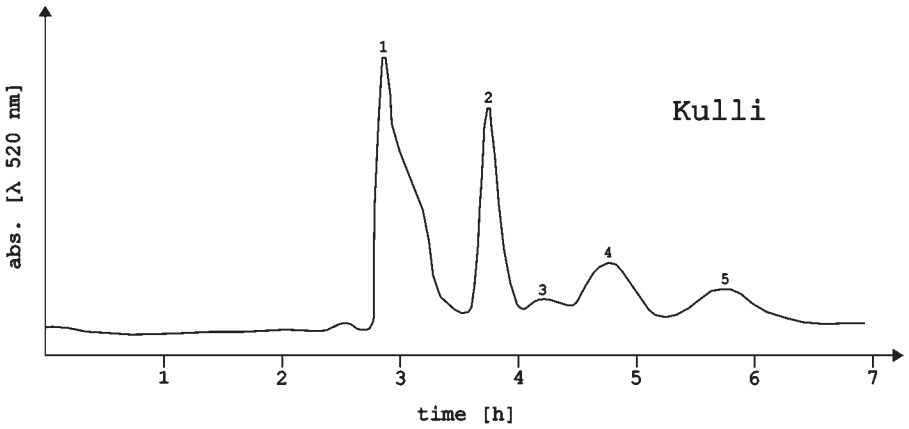

Figure 4. HSCCC separation of the purple corn cultivar Kulli monitored at 520 nm.

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REFERENCES

(1) Schwarz, M.; Hillebrand, S.; Habben, S.; Degenhardt, A.; Winterhalter, P. Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. *Biochem. Eng. J.* **2003**, *14*, 179–189.

(2) Kroon, P. A.; Williamson, G. Hydroxycinnamates in plants and food: current and future perspectives. *J. Agric. Food Chem.* **1999**, *79*, 355–361.

(3) Ralph, J. Hydroxycinnamates in lignification. *Phytochem. Rev.* **2010**, *9*, 65–83.

(4) Allerdings, E.; Ralph, J.; Steinhart, H.; Bunzel, M. Isolation and structural identification of complex feruloylated heteroxylan side-chains from maize bran. *Phytochemistry* **2006**, *67*, 1276–1286.

(5) Ishii, T.; Hiroi, T.; Thomas, J. R. Feruloylated xyloglucan and *p*-coumaroyl arabinoxylan oligosaccharides from bamboo shoot cell-walls. *Phytochemistry* **1990**, *29*, 1999–2003.

(6) Ishii, T. Structure and functions of feruloylated polysaccharides. *Plant Sci.* **1997**, *127*, 111–127.

(7) Bunzel, M. Chemistry and occurrence of hydroxycinnamate oligomers. *Phytochem. Rev.* **2010**, *9*, 47–64.

(8) Ralph, J.; Bunzel, M.; Marita, J. M.; Hatfield, R. D.; Lu, F.; Kim, H.; Schatz, P. F.; Grabber, J. H.; Steinhart, H. Peroxidase-dependent cross-linking reactions of *p*-hydroxycinnamates in plant cell walls. *Phytochem. Rev.* **2004**, *3*, 79–96.

(9) Bily, A. C.; Burt, A. J.; Ramputh, A.; Livesey, J.; Regnault-Roger, C.; Philogène, B. R.; Arnason, J. T. HPLC-PAD-APCI/MS assay of phenylpropanoids in cereals. *Phytochem. Anal.* **2004**, *15*, 9–15.

(10) Jing, P.; Noriega, V.; Schwartz, S. J.; Giusti, M. M. Effects of growing conditions on purple corncob (*Zea mays* L.) anthocyanins. *J. Agric. Food Chem.* **2007**, *55*, 8625–8629.

(11) Cevallos-Casals, B. A.; Cisneros-Zevallos, L. Stoichiometric and kinetic studies of phenolic antioxidants from Andean purple corn and red-fleshed sweetpotato. *J. Agric. Food Chem.* **2003**, *51*, 3313–3319.

(12) Toyoshi, T.; Kohda, T. Antihypertensive activity of purple corn color in spontaneously hypertensive rats. *Foods Food Ingredients J. Jpn.* **2004**, *209*, 671–679.

(13) Tsuda, T.; Horio, F.; Uchida, K.; Aoki, H.; Osawa, T. Dietary cyanidin 3-*O*-*β*-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *J. Nutr.* **2003**, *133*, 2125–2130.

(14) Aoki, H.; Wada, K.; Kuzo, N.; Ogawa, Y.; Koda, T. Inhibitory effect of anthocyanin colors on mutagenicity induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Foods Food Ingredients J. Jpn.* **2004**, *209*, 240–246.

(15) Hagiwara, A.; Miyashita, K.; Nakanishi, T.; Sano, M.; Tamano, S.; Kadota, T.; Koda, T.; Nakamura, M.; Imaida, K.; Ito, N.; Shirai, T. Pronounced inhibition by a natural anthocyanin, purple corn color, of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-associated colorectal carcinogenesis in male F344 rats pretreated with 1,2-dimethylhydrazine. *Cancer Lett.* **2001**, *171*, 17–25.

(16) Moreno, Y. S.; Sánchez, G. S.; Hernández, D. R.; Lobato, N. R. Characterization of anthocyanin extracts from maize kernels. *J. Chromatogr. Sci.* **2005**, *43*, 483–487.

(17) de Pascual-Teresa, S.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. LC-MS analysis of anthocyanins from purple corn cob. *J. Sci. Food Agric.* **2002**, *82*, 1003–1006.

(18) Fossen, T.; Slimestad, R.; Andersen, Ø. M. Anthocyanins from maize (*Zea mays*) and reed canarygrass (*Phalaris arundinacea*). *J. Agric. Food Chem.* **2001**, *49*, 2318–2321.

(19) Cuevas Montilla, E.; Hillebrand, S.; Butschbach, D.; Baldermann, S.; Watanabe, N.; Winterhalter, P. Preparative isolation of anthocyanins from Japanese purple sweet potato (*Ipomoea batatas* L.) varieties by high-speed countercurrent chromatography. *J. Agric. Food Chem.* **2010**, *58*, 9899–9904.

(20) Hillebrand, S.; Naumann, H.; Kitzinski, N.; Köhler, N.; Winterhalter, P. Isolation and characterization of anthocyanins from blue-fleshed potatoes (*Solanum tuberosum* L.). In *Potato III. Food 3 (Special Issue 1)*; Yee, N., Bussel, W., Eds.; Global Science Books: Miki cho, Japan, 2009; pp 96−101.

(21) Winterhalter, P. Application of countercurrent chromatography (CCC) to the analysis of natural pigments. *Trends Food Sci. Technol.* **2007**, *18*, 507–513.

(22) Cevallos-Casals, B. A.; Cisneros-Zevallos, L. Stability of anthocyanin-based aqueous extracts of Andean purple corn and red-fleshed sweet potato compared to synthetic and natural colorants. *Food Chem.* **2004**, *86*, 69–77.

(23) Pedreschi, R.; Cisneros-Zevallos, L. Phenolic profiles of Andean purple corn (*Zea mays* L.). *Food Chem.* **2005**, *100*, 956–963.

(24) Eichhorn, S.; Winterhalter, P. Anthocyanins from pigmented potato (*Solanum tuberosum* L.) varieties. *Food Res. Int.* **2005**, *38*, 943–948.

(25) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

(26) Robbins, R. J. Phenolic acids in foods: an overview of analytical methodology. *J. Agric. Food Chem.* **2003**, *51*, 2866–2887.

(27) Dobberstein, D.; Bunzel, M. Separation and detection of cell wall-bound ferulic acid dehydrodimers and dehydrotrimers in cereals and other plant materials by reversed phase high-performance liquid chromatography with ultraviolet detection. *J. Agric. Food Chem.* **2010**, *58*, 8927–8935.

(28) Boutigny, A.-L.; Atanasova-Pénichon, V.; Benet, M.; Barreau, C.; Richard-Forget, F. Natural phenolic acids from wheat bran inhibit *Fusarium culmorum* trichothecene biosynthesis *in vitro* by repressing *Tri* gene expression. *Eur. J. Plant Pathol.* **2010**, *127*, 275–286.

(29) Al-Weshahy, A; Rao, A. V. Isolation and characterization of functional components from peel samples of six potatoes varieties growing in Ontario. *Food Res. Int.* **2010**, *42*, 1062–1066.

(30) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grains*; CRC Press: Boca Raton, FL. 1993; p 265.

(31) Aoki, H.; Kuze, N.; Kato, Y. Anthocyanins isolated from purple corn (*Zea mays* L.). *Foods Food Ingredients J. Jpn.* **2002**, *199*, 41–45.

(32) González-Paramás, A. M.; Lopes da Silva, F.; Martín-López, P.; Macz-Pop, G.; González-Manzano, S.; Alcalde-Eon, C.; Pérez-Alonso, J. J.; Escribano-Bailón, M. T.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. Flavanol−anthocyanin condensed pigments in plant extracts. *Food Chem.* **2006**, *94*, 428–436.