Characterization of Anthocyanin Extracts from Maize Kernels

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Abstract

The aim of the present work is to characterize the pigments present in the kernel of four native maize varieties related to the races Arrocillo, Cónico, Peruano, and Purepecha to determine their possible use as natural dyes. Total anthocyanin content is determined by a conventional spectrophotometric method, and anthocyanin analysis is done by high-performance liquid chromatography. The stability of the pigment at pH is also evaluated. The four maize samples contained anthocyanin in both the pericarp and aleurone layer. Total anthocyanin content among samples ranged from 54 mg/100 g of sample to 115 mg/100 g of sample. Anthocyanin profiles are almost the same among the four samples. Differences are observed only in the relative percentage of each anthocyanin. The anthocyanins identified are cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-(6" malonylglucoside), and cyanidin-3-(3",6"-dimalonylglucoside). Anthocyanin extracts showed similar behavior in solutions with different pH. From pH 1–6 λ_{max} values are maintained almost constant; however, above this pH value, a marked increase is observed in the bathochromic shifts, but the bluish color did not continue to change above pH 8.

Introduction

Anthocyanins are the most important group of hydrosoluble compounds, and they are responsible for the red, purple, and blue colors seen in flowers, fruits, and other plant parts. For centuries, these compounds have been consumed by man with no evident harmful effect (1). Because of their brilliant colors and anti-inflamatory and antioxidant properties (2), anthocyanins are an important alternative for the substitution of synthetic dyes that have been banned in food because they have been associated with degenerative diseases.

Several sources of anthocyanins are being studied (3,4) to find one that is inexpensive and contains dominant anthocyanins of the acylated type, which are more stable under differing conditions of pH (5). The anthocyanins of maize are both nonacylated and acylated. In the vegetative structures of this gramineous plant, nonacylated type anthocyanins have been identified: cyanidin-3-glucoside, pelargonidin-3-glucoside, and peonidin-3-glucoside (6); among the acylated anthocyanins, cyanidin-3-malonylglucoside and cyanidin-3-dimalonylglucoside have been reported (7). The complete structure of these acylated anthocyanins, with the precise location of the points where the acyl radicals of sugar are joined, was obtained using techniques such as NMR in extracts of anthocyanins obtained from the maize tassel. In these extracts were also found peonidin 3(6" malonylglucoside) and peonidin-3-dimalonylglucoside (8). In the maize kernel there are reports of cyanidin-3-glucoside (9), and recently, the presence of pelargonidin-3-(5" malonylglucoside) has been reported in a commercial extract obtained from the ear of purple maize kernels (10). In kernels of blue maize, the anthocyanins are derived mainly from cyanidin and malvidin, the former being dominant, although in the kernels of red maize they are derived from pelargonidin, cyanidin, malvidin, and an unidentified aglycon (11).

The color of the maize kernels is indicative of the total anthocyanin content. In blue maize this can account for up to 62.7 mg/100 g of flour, though in purplish-red maize it varies between 8.7 and 61.0 mg/100 g of flour. Purplish-red maize kernels, with pigment in both the pericarp and the aleurone layer, contain the highest concentration of anthocyanins, with values of up to 141.7 mg anthocyanins/100 g of sample (11).

Anthocyanins are compounds whose color is easily affected by several factors; among these, pH is considered the most important. The color of the compounds varies (12) in a solution, depending on the pH of the solution.

Pigmented kernels are characteristic in most of the 41 maize races found in Mexico (13); among these are intense dark red maize varieties that are a possible source of natural dyes. However, it is not known whether the anthocyanins that give them these colors are the same as those described in other structures of this gramineous plant. Thus, this study was conducted to quantitate the total content of anthocyanins and identify the type of anthocyanins present in the kernels of four varieties of dark red maize. Also, the stability of the anthocyanin extracts in different pH values were assessed.

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Experimental

Plant material

The maize varieties used were from collections of the races Arrocillo, Cónico, Peruano, and Purepecha. The varieties of these races were selected for their intense purplish-red kernels with pigment in both the pericarp and aleurone layer (Figure 1, see page 3A). Because the samples include several varieties belonging to the same race, they are referred to by the race name to simplify reference.

Kernel color

A Hunter Lab miniscan XEPLUS colorimeter (Mod. PL50, Hunter Associates Laboratory Inc., Reston, VA) was used to obtain the values of L*, a*, and b* after calibrating with the white and black tiles. L* is a measure of lightness from completely opaque (0) to completely transparent (100); a* is a measure of redness (+a* = redness and –a* = greenness) and b* is a measure of yellowness (+b* = yellowness and –b* = blueness). The measurement was performed on samples of kernels placed tip-down on a bar of molding clay, simulating kernels on an ear. Using the values measured for a* and b*, the hue or angle (q) was calculated using the expression arctan b*/a*. Chroma was obtained using the formula $[(a^*)^2 + (b^*)^2]^{0.5}$.

Total anthocyanins

This was determined in flour samples obtained from degermed kernel, pericarp, and endosperm. The kernel was degermed and ground in a cyclonic type mill (UDY, Tecator, Hoganas, Sweden). The pericarp was separated manually from the kernel with a scalpel. Endosperm and pericarp were ground separately under the same conditions as the degermed grain.

To quantitate total anthocyanins in the degermed kernel and endosperm, 10 g of flour was weighed; for the pericarp, only 2 g was used. The extractive solvent was a mixture of methanol–acetic acid–water (10:1:9, v/v/v), with which three successive extractions were performed at room temperature (± 24°C) and agitated for 24 h in a horizontal shaker. Total anthocyanins were quantitated by a conventional spectrophotometric method (10) at 520 nm. The analyses were performed twice for each sample.

Anthocyanin extraction and purification

The anthocyanins in flour of the degermed kernel were extracted from a 5-g sample with 25 mL of a mixture of 1% trifluoracetic acid in methanol for 24 h at 5°C. The sample was filtered with Whatman No. 4 filter paper and an aliquot of 5 mL of the filtered liquid was poured into an Amberlite XAD-7 column (Whatman, Maidstone, U.K.), which was washed with 5% acetic acid and eluted with methanol acidified with 5% acetic acid (10). A second purification was performed using a Sephadex LH-20 column (Sigma, St. Louis, MO) as described by Fossen et al. (8).

High-performance liquid chromatography anthocyanin analysis

Agilent (HP) model 1100 equipment, comprising a manual injector, quaternary pump, and UV–vis detector (Agilent Technologies, Palo Alto, CA), was used. The analytical column used was an ODS-Hypersil (Thermo, Bellefonte, pA) (200- × 5-mm, 5-µm particle size). Analysis was performed using HCOOH–H_2O (1:9) (A) and HCOOH–H_2O–MeOH (1:4:5) (B) as the solvents, under the following gradient system: a linear gradient from 10% B to 100% B for 17 min, isocratic elution for the next 4 min (100% B), followed by a linear gradient from 100% B to 10% B for 1 min (8). The solvents used were high-performance liquid chromatography (HPLC) grade and were previously filtered with 0.2-µm nylon membranes (Millipore, Bedford, MA). The samples were filtered through a 0.45-µm syringe filter (Millipore, Bedford, MA) before they were injected. The volume of 20 µL was injected and a flow rate of 1.2 mL/min was used with a running time of 22 min. Anthocyanins were detected at 520 nm.

Alkaline hydrolysis

To determine whether the anthocyanins were of the acylated type, alkaline hydrolysis was performed using 1 mL of purified anthocyanin extract with Amberlite XAD-7, to which 10% KOH was added until the red color changed to blue. The mixture was left undisturbed for 10 min in a dark place, and later HCl 5M was added until the red color returned. The sample obtained was poured into a decanter funnel with a similar amount of diethylether to separate the anthocyanins from the acylated residues released during hydrolysis. The organic phase was concentrated by drying at reduced pressure. The sample was redissolved with a small amount of HCl 0.01M and later filtered through a Millex 0.45-µm syringe filter (Millipore) for injection into the HPLC (10).

Acid hydrolysis

Two milliliters of anthocyanin extract, previously purified with Amberlite XAD–7 resin, were mixed with 2 mL of HCl 6M, and the mixture was boiled for 40 min in a 50-mL Erlenmeyer flask. This sample was cooled and later dried by reduced pressure. It was then redissolved with a small amount of HCl 0.01M (10) and filtered through a 0.45-µm syringe filter before injection into the HPLC. Analysis was conducted under the same conditions as for alkaline hydrolysis.

Identification of anthocyanins

Anthocyanins were identified using commercial standards (Polyphenols Laboratories, NW, Sandnes, Norway) and by comparing the present results with those reported in the literature (8).

Stability of anthocyanins in differing pH

Stability was measured by obtaining the maximum values of absorbance of each sample in different pH values and assessing color changes of the solutions. Buffer solutions with different pH values were prepared, covering the range of 1 to 9. Two mg of the mixture of anthocyanins was mixed with 20 mL of each buffer solution, and scans were performed from 200 to 700 nm in a Milton Roy UV–vis spectrophotometer (Milton Roy, Rochester, NY). After scanning, the color of each of the samples was measured in terms of L*, a*, and b* using a Hunter Lab colorimeter. With the values of a* and b*, hue angle (q) was calculated using the expression arctan b*/a*. Chroma of each treatment was calculated using the formula $[(a^*)^2 + (b^*)^2]^{0.5}$.

Results and Discussion

Kernel color and total anthocyanin content

The naked-eye color of the kernel samples analyzed was purplish-red. The Hunter Lab parameters of the color of the samples indicated dark-colored kernels, given by the low L* values, hue indicated intense red. The sample with greatest purity of color was Peruano, having a lower scale of gray tones, with a chroma value of 4.98, compared with that of Arrocillo with a chroma of 2.00 (Table I).

The pigment was found in the aleurone layer and pericarp. Although the naked-eye color of the kernel and location of the pigment in the four maize varieties were the same, the content of anthocyanins varied. The sample with the highest content in the degermed kernel was Arrocillo, with 115.05 mg of anthocyanins/100 g of sample. The sample with the lowest content was Cónico, with only 54.00 mg anthocyanins/100 g of sample (Table II). The statistical analysis using Student *t*-test showed that the concentration of anthocyanin in Arrocillo kernels was significantly different [(a = 0.01, and $(n_1 + n_2 - 2) = 2$ freedom degree] from that in Cónico, Purepecha, and Peruano.

The highest concentration of anthocyanins in the pericarp was found in Peruano and Arrocillo. In the aleurone layer, anthocyanins were found in low concentrations in the four maize samples, especially in Peruano. Considering the possible use of maize kernels as a source of dyes, it is important that the greatest quantity be found in the pericarp because, as the outer covering of the seed, it is easier to separate from the rest of the kernel. The concentration of anthocyanins in the pericarp kernels of the maize samples evaluated is higher than that reported in purple grape skins of different varieties (14,15).

Table I. Color of Maize Kernels of the Samples Analyzed in Terms of L*, Hue, and Chroma

Sample	L*	Hue	Chroma
Arrocillo	16.08	10.37	2.00
Cónico	11.57	8.29	2.31
Peruano	14.10	11.87	4.98
Purepecha	11.16	15.45	2.88

Table II. Total Anthocyanin Content in the Degermed Whole Grain, Pericarp, and Endosperm of Four Purplish-Red Maize Races

Maize races	Degermed kernel	Pericarp	Endosperm
Arrocillo	115.0	1473.0	26.8
Cónico	54.0	504.0	23.6
Peruano	76.5	1524.4	4.2
Purepecha	76.6	812.8	21.3

* Values are means of two measurements and are expressed in mg of anthocyanins/100 g sample.

Analysis of anthocyanins and anthocyanidins

The chromatographic profile of anthocyanins in the kernels of the four maize samples was similar, with variation only in the proportion of each anthocyanin (Figure 2). The number of anthocyanins in the analyzed samples was between 8 and 10; of these, those of shorter retention time (< 11.4 min) were the nonacylated compounds, but those of longer retention time were those of the acylated type. This was determined by the HPLC analysis of a sample of anthocyanins after subjecting them to alkaline hydrolysis with KOH. Only four peaks of retention times shorter than 11.5 min were observed. Three of the anthocyanins were identified as cyanidin-3-glucoside, pelargonidin-3-glucoside, and

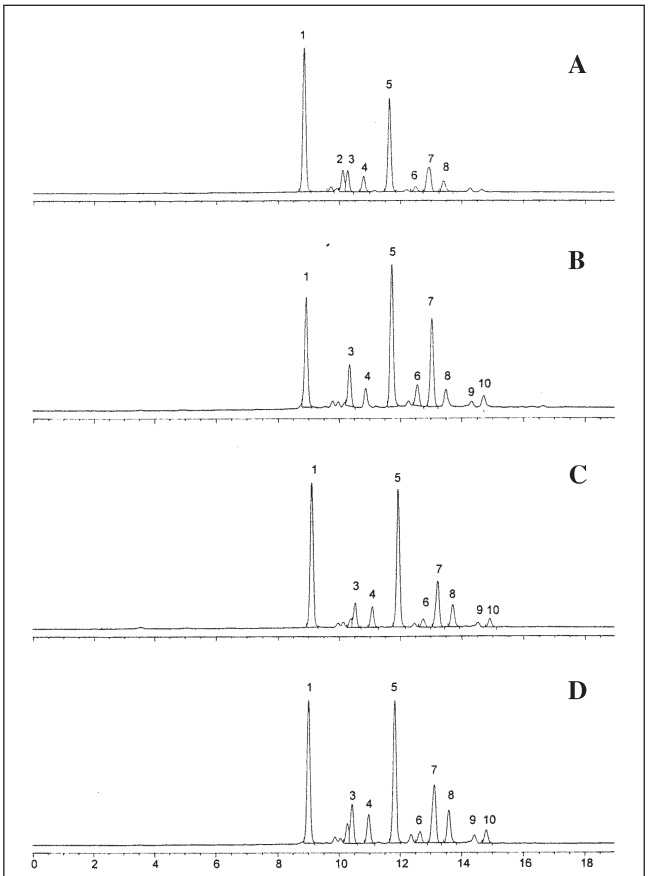

Figure 2. Chromatograms of HPLC analysis of the anthocyanins in purple-red maize kernels of Arrocillo (A), Cónico (B), Peruano (C), and Purepecha (D). The anthocyanins identified are cyanidin-3-glucoside (1), pelargonidin-3-glucoside (3), peonidin-3-glucoside (4), cyanidin-3-(6" malonylglucoside) (5), and cyanidin-3-(3",6" dimalonyl glucoside) (7). Anthocyanins 2, 6, 8, 9, and 10 were not identified.

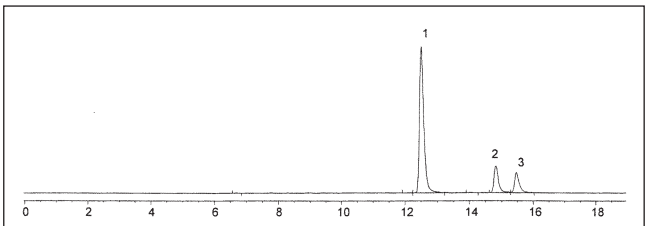

Figure 3. Chromatogram of HPLC analysis of the anthocyanidins (aglycons) from kernels of Arrocillo maize: Cyanidin (1), Pelargonidin (2), and Peonidin (3).

peonidin-3-glucoside using commercial standards. These three anthocyanins have been previously reported in ears of purple maize (10).

The acylated anthocyanins cyanidin-3-(6" malonylglucoside) and cyanidin-3-(3",6" dimalonyl glucoside) were identified in the maize samples by comparing the present results with those obtained by Fossen et al. (8) with maize tassel. The relative percentage of acylated anthocyanins in degermed kernels of the studied samples was found to be between 55.2% and 63.3%, with the exception of Arrocillo with 42% (data not shown). These results show that this relative percentage varies among the different maize races. In maize leaves and flowers, the percentage of acylated anthocyanins has been reported at 40% (8). The acylated anthocyanins are more stable to pH changes (5), and their presence in the maize extracts could help with their stability.

Table III. Relative Percentage of the Anthocyanidins from Kernels of Arrocillo, Cónico, Peruano, and Purepecha Maize

	Relative percentage of anthocyanidins (%)		
Maize race	Cyanidin	Pelargonidin	Peonidin
Arrocillo	73.8	14.3	12.0
Cónico	86.9	2.7	10.4
Peruano	79.2	6.5	14.3
Purepecha	73.1	10.8	16.1

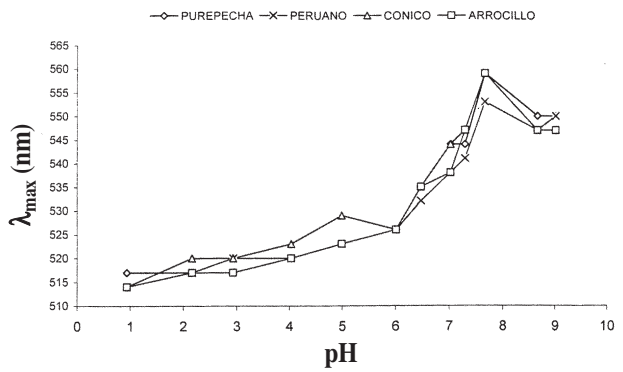

Figure 4. Maximum absorption values of anthocyanin extracts obtained from four maize samples in different pH.

The anthocyanins of the four samples studied are derived from only three anthocyanidins: cyanidin, pelargonidin, and peonidin. The prototype chromatogram of the samples analyzed is shown in Figure 3. In the four samples, the anthocyanidin present in greatest proportion was cyanidin, which was highest in Cónico, followed by Peruano. These two samples also had the lowest proportions of pelargonidin (Table III).

Stability of the anthocyanins in different pH

Light absorbance by the four extracts in different pH was similar: the values of λ_{max} remained with no major changes up to pH 6. However, above this pH there was a marked increase, reaching values of up to 559 nm, and falling again when the pH reached 7.66 (Figure 4). These results coincide with those reported by Cabrita et al. (16) for cyanidin-3-glucoside, in terms of changes in the λ_{max} values in solutions with different pH. Changes in λ_{max} values result from the modification of the chemical structure of the different anthocyanins that make up the mixture, as an effect of pH (12).

The extracts exhibited their purest color with the highest chroma values when pH was 1, with the exception of Arrocillo, with a highest chroma value found at pH 2.34. The color of all the extracts at this pH was very bright red-orange, generally corresponding to a dominant flavilium form structure (12). When pH was 2.97, significant changes ($p \leq 0.05$) in hue and chroma occurred in Cnico and Purepecha extracts, with less bright colors. Although in Arrocillo and Peruano, hue and chroma remained, with almost no significant change (Table IV).

Table IV. Color of the Anthocyanin Extracts in Terms of Chroma and Hue at Different pH*

	Cónico		Peruano		Arrocillo		Purepecha	
pH	Hue	Chroma	Hue	Chroma	Hue	Chroma	Hue	Chroma
1	13.1g	13.2a	18.5d	18.5[a]	16.9c	15.3a	19.2e	20.6a
2.34	12.9g	11.3ab	19.2d	15.7ab	16.9c	17.7a	16.2e	15.4b
2.97	10.2gh	10.5bc	19.1d	17.1[a]	15.9c	16.7a	11.4efg	14.6bc
3.99	3.0hi	5.6ef	14.2d	10.2c	9.2c	11.7b	13.9ef	18.8a
5.04	1.5i	8.1d	8.1d	12.9bc	2.7c	8.0c	1.4g	10.9d
6.1	0.8i	11.8ab	9.7d	5.8d	9.0c	8.7c	3.3fg	13.2bcd
6.49	348.3bc	6.5de	11.9d	12.3c	0.8C	7.8c	357.9a	11.0d
7	356.9a	12.3ab	7.6d	3.3de	353.3a	3.3d	353.8a	11.8cd
7.3	351.7ab	7.7de	12.7d	3.2de	350.8a	2.6d	352.6a	10.4d
7.71	342.9c	8.4cd	354.9a	1.7e	300.2b	1.4d	340.1bc	5.1e
8.14	297.8d	4.3fg	290.6c	0.9e	347.1a	2.8d	299.5d	2.1f
8.7	278.3f	2.4gh	278.2c	0.5e	345.4a	1.9d	349.1ab	3.7ef
9.32	288.2e	1.9h	322.6b	0.8e	350.7Aa	2.4d	335.3c	1.8f
MSD[†]	8.1	2.2	18.2	3.2	18.4	2.6	10.9	2.9

* Similar letters within columns indicates that values are not significantly different, (Tuckey test, $r \leq 0.05$).
[†] MSD = minimum significant difference.

When pH was neutral, the extracts from Cónico, Purepecha, and Arrocillo had a reddish-purple color, with the hue values in the fourth quadrant, though the sample from Peruano maintained an orange-red tone. Above this pH, all of the extracts had unpleasant grayish tones.

As pH increased from 2.97 up to 6.1, the extracts' red color decreased in brightness and took on a reddish-purple tone, which corresponds to lower hue and chroma values. An exception to this pattern was the Peruano extract, which maintained its hue value in the first quadrant of the Cartesian chart with reds tones up to pH 6.49. These changes of color were caused by a change in equilibrium of species of anthocyanins from the intensely red flavilium cation toward the colorless carbinol pseudobase and purple quinonoidal species (12).

In summation, although it was expected that the similar profiles of the anthocyanins of the four

extracts would result in similar color changes as an effect of pH, it was found that at least one of the samples had a different reaction even when exposed to solutions with the same pH. This implies that the color expressed by the mixtures was also influenced by the presence of copigments, as has been mentioned by other authors (17).

Conclusion

The analyzed samples had purplish-red kernels, and pigment was found in the aleurone layer and the pericarp. The sample with the highest total content of anthocyanins was Arrocillo, with 115.05 mg of anthocyanins/100 g of sample, though the lowest content was found in Cónico, with just 54.00 mg of anthocynins/100 g of sample. The chromatographic profiles of anthocyanins in the kernels of the four samples analyzed were similar, varying only the proportion of each anthocyanin.

The maize races Cónico, Peruano, and Purepecha had a higher proportion of acylated anthocyanins than the Arrocillo maize race. The anthocyanins of the kernel of the maize races analyzed are derived from only three anthocyanidins: cyanidin, pelargonidin, and peonidin; cyanidin was found in greater proportion. The four extracts maintain a consistent orange-red color within the pH range of 1 to 4. When pH is neutral, the color of the extracts changed to a dark red tone, with the exception of Peruano, which remained orange-red but with less purity of color.

The use of these extracts as food coloring in acidic and medium-acidic foods, such as yogurt and some sour candies, may be a promising alternative to synthetic dyes.

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