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Fernanda; Ruíz López, Mario Alberto; Navarro Ocaña, Arturo; Vargas Radillo, J. Jesús
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ANTHOCYANINS FROM ROSE MAIZE (*Zea mays* L.) GRAINS

Lucía Barrientos Ramírez, Hilda E. Ramírez Salcedo, María Fernanda Fernández Aulis, Mario Alberto Ruíz López, Arturo Navarro Ocaña and J. Jesús Vargas Radillo

SUMMARY

Here we present the anthocyanin composition of grains of rose corn (*Zea mays* L.). Extraction was performed using methanol:water:HCl in an ultrasound bath for 20min. Anthocyanins were isolated from maize using the combination of column chromatography and HPTLC plates, whereas HPLC-UV/Vis and HPLC-MS were used for analysis and identification. Compounds found include the glycosides Cyanidin,

Peonidin, and Pelargonidin, while HPLC profile revealed that the most abundant anthocyanin was Cyanidin-3-(6-malonyl-glucoside), a malonic derivative of Cyanidin. The results increase available data regarding the presence of these compounds in varieties of pigmented corn grains, and thus help to promote the consumption of these varieties for their beneficial health effects.

Introduction

The immediate predecessor of domesticated maize, the *Teocintle*, is considered native of Mesoamerica and, thus, Mexico is considered to be the origin of maize (*Zea mays* L., Poaceae family). Fifty nine of the 220-300 races of maize found in the American continent grow in Mexico (Sánchez *et al.*, 2000). In addition to white corn, there are over 45 races of pigmented maize (Salinas-Moreno *et al.*, 2005). Annual maize yield in 2014 was $\sim 23.27 \times 10^6$ t of grain, produced on $\sim 7.4 \times 10^6$ ha, representing an average yield of $3.14 \text{ t} \cdot \text{ha}^{-1}$ (SIAP, 2014). The Mexican state of Jalisco is the second largest national producer of corn, and harvested 3.47×10^6 t/year for a yield of $6.42 \text{ ton} \cdot \text{ha}^{-1}$, constituting 15% of national production (SIAP, 2014).

Anthocyanins, a water-soluble polyphenol, comprise one

or more sugar molecules (glycosylated type), and may also contain acyl radicals in the form of aliphatic organic acid derivatives linked to sugar (acylated type) which show greater stability at different pH levels (Eng *et al.*, 2017). Moreover, they show substantial bioactivity, mainly as antioxidants (Yang and Shai, 2011). These metabolites exhibit health benefits, promoting reduction of inflammation (He and Giusti, 2010); with properties such as prevention of the chronic diseases diabetes and obesity (Li *et al.*, 2015) and cancer (Chen *et al.*, 2015), among other.

The Mexican races of maize present kernels with white, yellow, pink, red, blue, purple and black coloration (Bello-Pérez *et al.*, 2016). The color is related to the amounts and types of anthocyanins. The pigmented maize varieties are desirable as food, as well as

for cosmetic or pharmaceutical applications (Ignat *et al.*, 2011). They also have functional properties, since they cause a slow digestion of starch that has been associated with beneficial health effects like the control of hunger and an more evenly spaced food consumption (Bello-Pérez *et al.*, 2016). Bibliographic reports mention anthocyanin contents of $9\text{-}15.9 \text{ mg} \cdot \text{kg}^{-1}$ dry matter (DM) in white corn, $163.9 \text{ mg} \cdot \text{kg}^{-1}$ DM pink corn, 342.2 in blue corn, 1270 in red corn, 1277 in purple corn, and $5290 \text{ mg} \cdot \text{kg}^{-1}$ DM in black corn (Salinas-Moreno *et al.*, 2013; Escalante-Aburto *et al.*, 2016).

The rose western corn is a very productive variety, with cobs of 15 to 20cm in length, cylindrical shape, and 12 to 14 lines. The grain is jagged, pink or pink-red. This maize is included in the variety of cone shaped corn races.

Traditionally, it is used to make *tortillas*, *pozole*, *gorditas*, and sweet *atole* (Ron Parra *et al.*, 2006). The present study aims to assess the types and characteristics of anthocyanin metabolites in this corn variety, which is abundant in many regions, with large availability and interesting biological properties due to the presence of anthocyanin and other polyphenols.

Materials and Methods

Sample

Rose maize (*Zea mays* L.; National Registry N° 05046) was harvested in the summer (rainy season) in the period of June-August of 2015 in a property located in the town of Nextipac, Zapopan, Jalisco, Mexico ($20^{\circ}45'51''\text{N}$; $103^{\circ}31'44''\text{W}$; 1634masl). Maize is cultivated annually under the environmental conditions that

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Lucía Barrientos Ramírez. Doctor of Science in Biology, Universidad de Guadalajara (UdeG), Mexico. Professor, UdeG, Mexico. e-mail: lbarrien@dmcyp.cucei.udg.mx

Hilda E. Ramírez Salcedo. Doctoral student, BEMARENA, UdeG, Mexico. e-mail: hildaelisal8@hotmail.com.

María Fernanda Fernández Aulis. Doctoral student in Chemistry, Universidad Nacional Autónoma de México (UNAM). e-mail: mffaulis@hotmail.com

Mario Alberto Ruíz López. Doctor of Science in Biology, UNAM, Mexico. Professor, UdeG, Mexico. e-mail: mruiz@cucba.udg.mx

Arturo Navarro Ocaña. Doctor in Biotechnology, CCH-UNAM, Mexico. Professor, UNAM, Mexico e-mail: arturono@unam.mx

J. Jesús Vargas Radillo. Doctor in Biosystematics, Ecology and Natural and Agricultural Resources Management (BEMARENA), UdeG, Mexico. Pro-

fessor, UdeG, Mexico. Address: Departamento de Madera, Celulosa y Papel (DMCyP), UdeG. Zapopan, Jalisco, México, CP 45100, tel. (33)-36820110. e-mail: jvargasr@dmcyp.cucei.udg.mx.

ANTOCIANINAS DE GRANOS DE MAIZ (*Zea mays* L.) ROSA

Lucía Barrientos Ramírez, Hilda E. Ramírez Salcedo, María Fernanda Fernández Aulis, Mario Alberto Ruíz López, Arturo Navarro Ocaña y J. Jesús Vargas Radillo

RESUMEN

Se presenta la composición de antocianinas en granos de maíz *Zea mays* L., de la raza elotes occidentales tipo maíz rosa. La extracción de estas fue con un sistema metanol:agua:HCl, usando un baño ultrasonido por 20min, para posteriormente ser analizado por HPLC-DAD y HPLC-MS. Se identificaron glucósidos de Cianidina, Pelargonidina y Peonidina, siendo la Cianid-

ina-3-(6-malonilglucosido), un derivado malónico de la Cianidina, el compuesto más abundante de acuerdo con su perfil cromatográfico. Estos resultados aumentan el acervo de datos sobre estos compuestos presentes en las variedades de los granos pigmentados, y buscan fomentar el consumo de estas variedades por sus efectos benéficos en la salud.

ANTOCIANINAS DE GRÃOS DE MILHO (*Zea mays* L.) ROSA

Lucía Barrientos Ramírez, Hilda E. Ramírez Salcedo, María Fernanda Fernández Aulis, Mario Alberto Ruíz López, Arturo Navarro Ocaña e J. Jesús Vargas Radillo

RESUMO

Apresenta-se a composição de antocianinas em grãos de milho *Zea mays* L., da raça elote ocidental, tipo milho rosa. A extração destas foi realizada com um sistema metanol:água:HCl, usando um banho ultrassom por 20min, para posteriormente ser analisado por HPLC-DAD e HPLC-MS. Identificaram-se glicosídeos de Cianidina, Pelargonidina e Peonidina, sendo a Cianidi-

na-3-(6-malonilglucosídeo), um derivado malônico da Cianidina, o composto mais abundante de acordo com seu perfil cromatográfico. Estes resultados aumentam o acervo de dados sobre estes compostos presentes nas variedades dos grãos pigmentados, e buscam fomentar o consumo destas variedades por seus efeitos benéficos na saúde.

prevail in the area: average temperature 20-22°C; average rainfall 980mm (Climate-data, 2018; Sagarpa, 2015), applying traditional fertilization consisting of the incorporation of nitrogen and phosphorus, on a Regosol type soil (sandy-loam texture). About 30 pieces were sampled, obtaining the grains manually. A representative sample was evaluated for anthocyanin content as follows: the corn kernels were manually reeled off and washed, they were then pulverized in a hammer mill (Retsch GmbH) using a screen with 0.5mm perforations, and the obtained flour was dehydrated to constant weight in an oven at 40°C.

Extraction and purification by combined column chromatography

A schematic of the proposed method is illustrated in Figure 1. Anthocyanins were obtained with the ultrasound assisted extraction (UAE) procedure (Vinatoru, 2001). The flour ground from whole corn (10g) was extracted with 100ml (10:1 w/v) of 80% (v/v) me-

thanol acidified with 0.1% HCl, in an ultrasonic bath (Branson model 8892; 42kHz), for 20min. The extract was filtered and concentrated by rotoevaporation at <40°C and purified making use of a column (2.0x60cm) of amberlite (XAD-7HP, 20-60 mesh; Sigma-Aldrich). Amberlite is an acrylic resin, nonionic, moderately polar material that partially purified the anthocyanins based on polar/non-polar interactions (Wang *et al.*, 2014). The column was activated with 250ml of methanol at a flow rate of 1ml·min⁻¹ and so washed with 500ml of distilled water in order to achieve a greater adsorption of the organic compounds and, then the extracts were loaded onto a column and washed with abundant distilled water to remove most of sugars, organic acids, proteins and ions (Wang *et al.*, 2014). The anthocyanin pigment fraction was recovered from amberlite with 300ml of methanol:acetic acid (19:1 v/v), concentrated by rotary evaporator at <40°C, dried (Labkept in amber flasks at 4°C. This procedure was repeated sequen-

tially until there was no more separation of spots on the plate.

HPLC analysis

Crude extract was filtered through a 0.45µm membrane and analyzed using a Waters liquid chromatograph equipped with a 2707 autosampler, a 1525 binary pump, a 2478

UV/Vis detector (520nm) and a Hypersil Gold ODS column (250x4.4mm, 5µm). We used a gradient of solvent A (H₂O:ACN: HCOOH, 87:3:10) and solvent B (ACN), with the following elution program: 97% A and 3% B from 0-45min; 75% A and 25% B from 45-46min; 70% A and 30% B from 46-47min; and

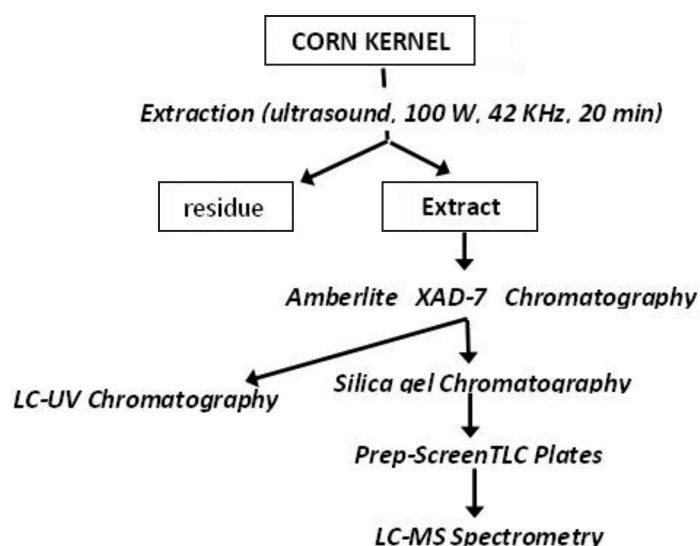


Figure 1. Isolation and fragmentation scheme of anthocyanins of corn grains.

97% A and 3% B from 47-56min. The flow rate was 1.0ml·min⁻¹, and the injection volume was 20μl.

MS conditions

Mass spectrometry was performed using an Agilent Technologies model 6430, series 1200, mass spectrometer equipped with a quadrupole mass filter and electrospray system ionization (ESI). Sample injection volume was 10μl; detection 520nm; dry gas (N₂), 12l·min⁻¹, dry temperature 400°C; capillary voltage 4.5kV. Analysis was performed in ion positive mode and scanning from 200 m/z to 1000 m/z.

Results and Discussion

The extracts were evaluated by chromatography. Preliminarily by TLC using typical standards for corn anthocyanin. Although there was no separation of compounds for the pink corn extract in the thin-film plate (line 1 in Figure 2), the spot agrees with the standards of Pelargonidin-3-glucoside and Cyanidin-3- (6-malonyl) glucoside. To achieve a greater elucida-

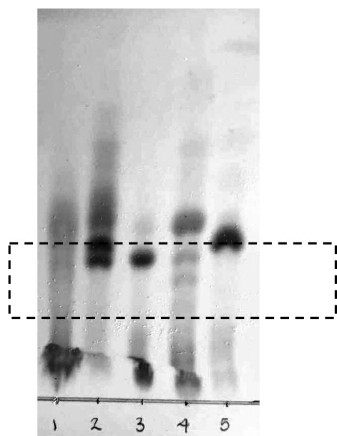


Figure 2. Crude extract Silica-TLC of pink corn compared with anthocyanin standards. 1: Rose corn kernel, 2: purple corn kernel, 3: Cyanidin-3-glucoside standard from jaboticaba, 4: Pelargonidin-3-glucoside standard from strawberry, 5: Cyanidin-3-(6 malonyl) glucoside standard from purple lettuce. Elution system: etil acetate: water: formic acid (75:20:10).

the crude extract of pink corn was processed by HPLC.

Figure 3 shows the HPLC profile of the corn anthocyanins, which exhibit several signals. The eight anthocyanins that were identified in this study are numbered, with peaks 4, 2, 8, 1 predominating by their height and area, due their greater relative abundance. Similar amount of signals (21 spots or fractions) were obtained by column-TLC, as shown in Figure 4 and Table I, some of them by the combination of spots with similar Rf. TLC is an analytical tool, simple, fast, cheap and frequently used as a technique for separation in natural products and organic chemistry, with low consumption of solvents. It allows the simultaneous analysis of several samples, it does not require special instrumentation, and combined with mass spectrometry is considered as useful tool in routine analytical methods (Ignat *et al.*, 2011).

The purified and recovered anthocyanins obtained from

TABLE I
Rf VALUES OF SPOTS OBSERVED IN THE THIN-LAYER CHROMATOGRAM

Fraction	Rf value
5	0.878
6	0.683, 0.816, 0.025, 0.683
7	0.480, 0.496, 0.880
8	0.513, 0.843, 0.565, 0.886
9	0.448, 0.480, 0.520, 0.600
10-13	0.458
14-15	0.450
16-17	0.420
20-24	0.432

Rf: retention/retardation factor

column-TLC were analyzed by LC-MS obtaining their fragmentation pattern. To identify the anthocyanins, we compared the peak information to previously reported data (Montilla *et al.*, 2011; Salinas-Moreno *et al.*, 2012; Zilic *et al.*, 2012), including the retention times (t_R) in HPLC, mass/charge (m/z), and characteristic fragmentation patterns for each molecule obtained by LC-ESI-MS. Known maize aglycones include Cyanidin (m/z 287), Pelargonidin (m/z 287) and

Peonidin (m/z 301). On the other hand, glycosides (anthocyanins) contain mainly glucose, or are further acylated with malonic acid.

The identification results are presented in Table II. Peak 4 is the most abundant, and shows a t_R of 18.3min, a molecular ion [M]⁺ of 535, and fragments of m/z 449 and 287, corresponding to Cyanidin-3-(6-malonyl-glucoside). Similarly, studies of purple corn kernels have reported that this anthocyanin is the most abundant (Salinas-Moreno *et al.*, 2012). Peak 1 (t_R 12.6min, m/z 449→287) correspond to Cyanidin-3-glucoside, which has been previously reported to be the most common anthocyanin in maize (Escalante-Aburto *et al.*, 2016). Peak 2 (t_R 14.4min, m/z 433→271) agrees with Pelargonidin-3-glucoside, peak 3 (t_R 16.0min, m/z 463→301) corresponds to Peonidin-3-glucoside and peak 5 (t_R 20.3min, m/z 549→463, 301) matches Peonidin-3-(6-malonyl-glucoside). Based on previous reports (Cuevas *et al.*, 2011) and fragmentation patterns, peaks 6, 7 and 8 would correspond, respectively, to doubly acylated Cyanidin, Pelargonidin and Peonidin with malonic acid moiety. These anthocyanidin (Cyanidin, Pelargonidin and Peonidin) are characteristic in magenta maize, which is the most studied group of grains, and includes maize with grain colors from pink to purple, including red corn (Salinas-Moreno *et al.*, 2012). Hence, the anthocyanins from western race corn variety rose (Table II) are coincident with those found in most of the purple-red maize: Cyanidin-3-glucoside, Pelargonidin-3-glucoside, Peonidin-3-glucoside, Cyanidin-3-(6-malonyl-glucoside), and Cyanidin-3-(3-6-dimalonyl-glucoside), with Cyanidin as the main aglycon (Salinas-Moreno 2005; Escalante-Aburto *et al.*, 2016). However, in maize with grains of rose hue Peonidin is the relatively more abundant aglycon, according to the intensity of the peaks in Figure 3.

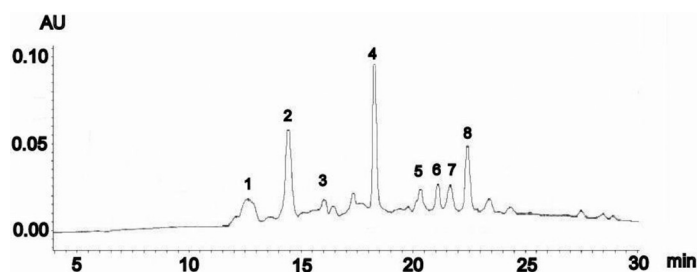


Figure 3. HPLC chromatogram (UV-detector at 520nm) of crude corn anthocyanin extracts obtained with MeOH:H₂O:HCl system solvent and cleaning through amberlite column. AU: absorbance units.

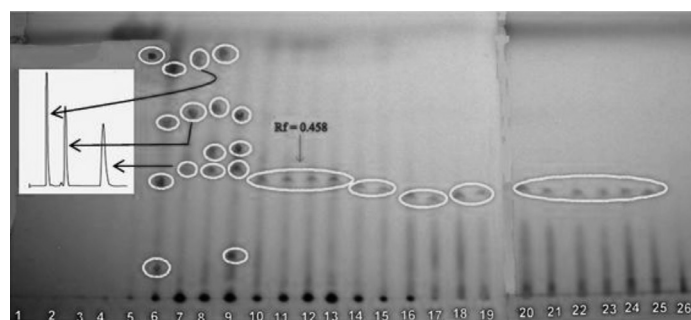


Figure 4. Silica TLC development of extracts from rose corn kernels visualized with UV light at 325nm. Each spot with different Rf is considered as a monomeric anthocyanin. Spots with similar Rf were combined.

TABLE II
MS AND CHROMATOGRAPHIC CHARACTERISTICS RELATIVE TO RETENTION TIME
AND FRAGMENTATION PATTERNS OF ANTHOCYANINS FOUND IN ROSE CORN
KERNELS

Peak	t _r (min)	Fragments [M-x] ⁺ (m/z)	Aglicon [A] ⁺	Compound	Molecular formula
1	12.6	287	287 [Cy] ⁺	Cy-3-Glc	C ₂₁ H ₂₁ O ₁₁
2	14.4	271	271 [Pg] ⁺	Pg-3-Glc	C ₂₁ H ₂₁ O ₁₀
3	16.0	301	301 [Pn] ⁺	Pn-3-Glc	C ₂₂ H ₂₃ O ₁₁
4	18.3	449, 287	287 [Cy] ⁺	Cy-3-(6-Mal-Glc)	C ₂₄ H ₂₃ O ₁₄
5	20.3	463, 301	301 [Pn] ⁺	Pn-3-(6-Mal-Glc)	C ₂₅ H ₂₅ O ₁₄

Cy: Cyanidin, Pg: Pelargonidin, Pn: Peonidin, Glc: glucoside, Mal: malonic acid, t_r: retention time, m/z: mass-to-charge ratio.

The lower intensity of color of this pink-red maize would indicate a lower content of anthocyanins as compared to blue maize (342mg anthocyanins/kg flour) and that of red maize (1270mg/kg flour), although it exhibits a similar profile.

Pigmented corn represents only 10% of the total corn production in Mexico, which indicates a low utilization considering that its nutritional content and nutraceutical properties represent a great opportunity for the development of new products, with new or better functional and nutritional characteristics (Bello-Pérez *et al.*, 2016). This and other pigmented corn has a large potential market. Nixtamalized maize products like *tortillas*, pregelatinized flours, chips, extruded breakfast cereals, and snack *totopos* obtained mainly from white and yellow corn, are used in countries, as diverse as China and Australia (Bello-Perez *et al.*, 2016). Also, due to its stability in acid medium anthocyanin could be an additive in fermented milk and yogurts (Cavalcante dos Santos *et al.*, 2017). From their brilliant colors, they are an alternative to obtain natural food colorants which would replace synthetic dyes that have been banned in food since they have been related to degenerative diseases (Salinas-Moreno *et al.*, 2005). Also, these pigments are potential pharmaceutical ingredients that provide various health benefits (Eng *et al.*, 2017). The information gathered could be useful to choose the most suitable maize for the food industry, according to its health benefits and nutraceutical value (Salinas-Moreno *et al.*, 2012).

Conclusions

This study aims to contribute to the knowledge of dominant acylated and non-acylated anthocyanins in the rose-red kernel maize variety, using a non-conventional extraction method, purification and fractionation by simple and sensitive chromatographic techniques (combined column chromatography - Prep-Screen TLC Plates), and identification of anthocyanins by a HPLC-MS system. We identified five anthocyanins from whole-grain flour made from rose corn. The applied chromatographic and spectroscopic methods allowed the identification of the anthocyanidins Cyanidin, Peonidin, and Pelargonidin, presents in this variety, as well as the most abundant anthocyanin, the Cyanidin-3-(6-malonyl-glucoside), a malonic derivative of Cyanidin. Since corn is native to Mesoamerica and is the basis of the Mexican diet, it is important to study the anthocyanins present in the various varieties of pigmented corn grains to promote their consumption for beneficial health effects. Products that contain anthocyanins, such as fruits and vegetables, are looked upon for their health benefits. Consumption of colored corn products could represent an important contribution in this regard.

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