Centro de Investigación en Alimentación y Desarrollo, A.C.

EVALUACIÓN DE COMPUESTOS FENÓLICOS DURANTE LA MADURACIÓN DE MANGO (*Mangifera indica* L., Ataulfo). UN ENFOQUE MOLECULAR Y BIOQUÍMICO

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EVALUACIÓN DE COMPUESTOS FENÓLICOS DURANTE LA MADURACIÓN DE MANGO (*Mangifera indica* L., Ataulfo). UN ENFOQUE MOLECULAR Y BIOQUÍMICO

Sinopsis

Varios estudios clínicos y epidemiológicos han demostrado que las frutas y verduras contienen compuestos bioactivos con actividad antioxidante que promueven beneficios para la salud. Estos compuestos pueden ser de diferentes clases químicas como los compuestos fenólicos, carotenoides y vitaminas. El mango (*Mangifera indica* L) es considerado una buena fuente de antioxidantes en la dieta, entre los que destacan el ácido ascórbico, los carotenoides y en especial los compuestos fenólicos que han demostrado diferentes propiedades para promover la salud, principalmente debido a su notable capacidad antioxidante. La presencia de compuestos bioactivos se ha relacionado con la prevención de enfermedades cardiovasculares, la aterosclerosis y en la disminución de riesgos asociados a algunos tipos de cáncer, entre otros beneficios. Por lo tanto, el consumo regular de mango podría proporcionar cantidades significativas de compuestos bioactivos para promover una vida sana en los consumidores.

El mango es una fruta popular y económicamente importante en varias partes del mundo, debido a sus excelentes propiedades sensoriales (color brillante, sabor dulce y delicioso) y su composición nutricional (vitaminas, minerales, fibra y fitoquímicos). Por otro lado, recientemente, se reportó que el mango 'Ataulfo' contiene el mayor contenido de compuestos fenólicos y capacidad antioxidante en comparación con variedades comerciales de mango (`Kent´, `Keitt´, `Haden´ y `Manila´), además de tener una mayor vida de anaquel. Se sabe que existen diferentes factores que pueden afectar la capacidad antioxidante, como son la variedad (genotipo), las condiciones agronómicas, manejo post-cosecha y el estado de madurez del fruto.

Aunque los compuestos fenólicos se han investigado en frutas tropicales como mango, existe una falta de conocimiento acerca de los cambios que ocurren de éstos compuestos durante el proceso de maduración. El estado nutricional de las frutas correlaciona bien con el período de conservación y la resistencia contra diferentes estreses. Sin embargo, los cambios de estos parámetros durante la maduración de mango 'Ataulfo' son desconocidos. Por lo tanto, en una de las etapas de este trabajo el objetivo fue evaluar cómo las etapas de maduración afectan los parámetros fisiológicos y de calidad, el contenido y la composición fenólica total y la capacidad antioxidante de fruta de mango 'Ataulfo'.

Para ello, se establecieron cuatro estados de madurez (EM), que se asignaron de acuerdo a una escala subjetiva de color (apoyada por parámetros fisiológicos) de la siguiente manera: EM1, que representa mango con una superficie amarilla del 0-10%; ME2, 20-30%; EM3, 70-80% y EM4, 100% color amarillo. Con esta evaluación se concluyó que el proceso fisiológico de maduración influye directamente en el contenido y la actividad antioxidante de los compuestos fenólicos en frutos de mango 'Ataulfo'. Además, el pico climatérico del fruto se observó en el EM3, que fue donde se observó el mayor valor de compuestos fenólicos totales y capacidad antioxidante **(Capítulo I).**

Aunque los compuestos fenólicos totales y actividad antioxidante fueron evaluados en mango ´Ataulfo´, existía una falta de conocimiento acerca de la composición específica y cambios de los principales compuestos fenólicos que participaban en la capacidad antioxidante durante la maduración de este fruto. Para la determinación de la actividad antioxidante de tejidos vegetales se deben tomar en cuenta las concentraciones totales y las concentraciones individuales de los compuestos fenólicos específicos, o en su caso los más abundantes. La actividad antioxidante en mango 'Ataulfo' se debe a las actividades individuales y combinadas de sus compuestos antioxidantes que actúan, en general, en forma sinérgica. Por lo tanto, fue conveniente evaluar la contribución individual de los fenoles a la actividad antioxidante total del fruto, con el fin de entender los cambios antioxidantes más reales que se producen durante la maduración. Por ello, en este trabajo se abordó como siguiente etapa la identificación, cuantificación y evaluación de la contribución individual antioxidante de los principales compuestos fenólicos que se encuentran en la pulpa de mango 'Ataulfo'

en las diferentes etapas de maduración. De acuerdo a los resultados obtenidos se concluyó de forma general que los ácidos fenólicos son los compuestos fenólicos predominantes, siendo ácido clorogénico el más abundante en la pulpa del fruto, seguido de ácido gálico. A su vez, el contenido de ácidos fenólicos aumentó conforme avanzaba la maduración, siendo el ácido gálico el que contribuyó en mayor medida a la capacidad antioxidante **(Capítulo II).**

Los ácidos fenólicos son antioxidantes que se encuentran en el centro de atención de la investigación clínica y epidemiológica, debido a que son componentes antioxidantes principales de las frutas y hortalizas. Se ha reportado que el consumo de estos ácidos fenólicos presentes en frutas y vegetales u otras matrices alimentarias, tiene una relación inversa con la incidencia de diversas enfermedades, y en este caso, ácidos como clorogénico y gálico podrían estar estrechamente relacionados con los beneficios a la salud de los consumidores. De acuerdo a la etapa anterior, se detectó que los compuestos fenólicos principales que se encuentran en orden de abundancia en la pulpa de mango 'Ataulfo' son ácido clorogénico, gálico, protocateico y vanílico. La relación entre los compuestos fenólicos bioactivos, su capacidad antioxidante y los beneficios para la salud están bien establecidos. Sin embargo, la información sobre los ácidos fenólicos y sus posibles interacciones sinérgicas o antagónicas para generar la actividad antioxidante es escasa.

Cada compuesto fenólico tiene una capacidad antioxidante diferente dependiendo de su estructura química, del número de grupos hidroxilo y su distribución. No se sabía si la contribución individual de cada fenólico en mango ´Ataulfo´ era aditiva, sinérgica o antagonista, incluso entre ellos. Para obtener un mejor conocimiento sobre las interacciones complejas en un sistema real, se tuvo como objetivo evaluar las actividades antioxidantes individuales y combinadas de esas moléculas fenólicas. Esto ayudó a proporcionar pistas importantes para esclarecer los mecanismos por los cuales estos fenoles mayoritarios ejercen su capacidad antioxidante en el fruto. Se concluyó que los cuatro ácidos fenólicos actúan de forma sinérgica, y solo en un caso se observó efecto antagónico, específicamente relacionado con ácido vanílico. Además, se observó que la mayor capacidad antioxidante la exhibe la molécula de ácido gálico, seguido de protocateico, clorogénico y finalmente vanílico **(Capítulo III).**

Después de examinar el escenario antioxidante que presentan los compuestos fenólicos durante la maduración de mango ´Ataulfo´; fue pertinente abordar otro aspecto del tipo estrictamente fisiológico. Sobre todo, preguntarnos cuales es el rol de las vías metabólicas que hacen que finalmente estén presentes dichos antioxidantes en la pulpa de mango.

La maduración de la fruta es un proceso regulado durante el desarrollo resultante de la coordinación de numerosos eventos bioquímicos y fisiológicos que ocurren en el tejido de la fruta que dan como resultado algunos cambios organolépticos. Los compuestos fenólicos juegan un papel vital para las plantas y son producidos por la vía de los fenilpropanoides. Estos compuestos contribuyen no solo a la pigmentación y sabor de la fruta, sino principalmente como mecanismo defensa contra cualquier agente de estrés que sea un riesgo para la célula vegetal (ataque de microorganismos patógenos, ataque de insectos, luz ultravioleta, estrés químico endógeno y exógeno), entre otras funciones metabólicas.

Si bien la ruta de los fenilpropanoides es esencial en todas las células vegetales, la regulación de la expresión de los genes que codifican para las enzimas de la biosíntesis de dichos compuestos, es particular de acuerdo al tipo de tejido. Además, la relación entre el proceso de maduración de la fruta y la biosíntesis de compuestos fenólicos es un tema complejo de abordar, y existe amplio debate al respecto hoy en día. Sin embargo, se han encontrado evidencias de que el proceso de maduración afecta directamente la ruta de los fenilpropanoides. Hay varias enzimas clave que están implicadas en la vía fenilpropanoide tales como la fenilalanina amonio liasa (PAL), ácido cinámico 4-hidroxilasa (C4H), *p*-cumarato 3-hidroxilasa (C3H), 4-cumarato CoA ligasa (4CL), entre otros tales como la Omethyltransferase (OMT). El papel de PAL es dirigir el flujo de carbono desde el aminoácido aromático L-fenilalanina (L-Phe) para la producción de 4-cumaroil-CoA. En el caso de C3H, su papel está implicado en la hidroxilación en el carbono 3 ' del anillo aromático en diversos productos intermedios de ácidos fenólicos. En este sentido, el papel de los genes *PAL* y *C3H* son necesarios para la biosíntesis de casi todos los compuestos fenólicos presentes en la naturaleza.

Por consiguiente, fue conveniente evaluar la expresión de dichos genes y la actividad de las enzimas codificadas en ellos con el fin de entender y/o estimar su

participación durante la maduración de la fruta del mango ´Ataulfo´. Con la evaluación de esta etapa, se determinó que la expresión *PAL* fue mayor al principio y al final de la maduración, lo que refleja que la activación del gen se genera en dos diferentes momentos metabólicos de la maduración del fruto de mango. En cuanto a la actividad enzimática PAL, en contraste con la expresión de *PAL*, la enzima es activa durante toda la maduración; con un aumento significativo en los últimos dos EM del fruto. La expresión no correlaciona con la actividad de la enzima, lo que sugiere que la regulación de la expresión génica de PAL se lleva a cabo a diferentes niveles en el flujo de la información genética. En cuanto a la actividad enzimática de C3H, se encontró una correlación positiva con la expresión *C3H*, ya que la actividad enzimática aumentó de RS1 a RS3, con una disminución significativa en la RS2. Además, la mayor actividad enzimática se observó en el EM3, lo que sugiere que la regulación de *C3H* es a nivel transcripcional **(Capítulo IV).**

Con base a los resultados obtenidos, se puede concluir que la pulpa de mango ´Ataulfo´ presenta de manera característica ácidos fenólicos como principal grupo de compuestos fenólicos. Que estos ácidos son los responsables de la mayor capacidad antioxidante hidrofílica exhibida por el fruto, y que éstos están actuando de una forma sinérgica. Además, el estado de madurez influye directamente en la biosíntesis de los compuestos fenólicos, asociándose la mayor presencia y actividad antioxidante al pico climatérico del fruto.

Por otro lado, se evidenció la relación de la expresión y de actividad enzimática de PAL y C3H, y el posible rol desempeñado por los genes *PAL* y *C3H* durante la maduración. Este estudio pone de relieve la naturaleza compleja sobre la regulación de la biosíntesis de los compuestos fenólicos en frutas tropicales como mango, al menos a nivel genético. Esperamos que este trabajo proporcione información valiosa para futuras investigaciones en la comprensión de los conceptos emergentes relacionada con la biosíntesis de fenilpropanoides. Además, fortalecer las bases sobre el entendimiento del comportamiento antioxidante de compuestos fenólicos durante la maduración de frutos tropicales.

I

EFFECT OF RIPENESS STAGE OF MANGO FRUIT (*Mangifera indica* L., cv. Ataulfo) ON PHYSIOLOGICAL PARAMETERS AND ANTIOXIDANT ACTIVITY

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Effect of ripeness stage of mango fruit (*Mangifera indica* L., cv. Ataulfo) on physiological parameters and antioxidant activity

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ABSTRACT

Many phenolic compounds influence the organoleptic quality of fruits and provide health benefits to consumers due to their antioxidant capacity. Since 'Ataulfo' mango has the highest phenolic content among other mango cultivars, the aim of this research was to investigate how the ripening stage affects their total phenolic content and antioxidant activity. Quality parameters, phenolic content and the antioxidant potential measured by DPPH and FRAP, of mango fruits of four ripening stages (RS) were determined. RS1, representing mango with yellow surface area of 0–10%; RS2, 11–40%; RS3, 41–70% and RS4, 71–100% yellow color. The quality parameters were significantly different ($P \leq 0.05$) in fruits of different RS, except for firmness and pulp color that were similar in fruits from RS3 and RS4. Mango fruits from RS2 and RS3 accumulated the highest phenol content (174 mg EAG/100 g FW) and antioxidant capacity measured by DPPH (93% inhibition). In general, the antioxidant capacity in fruit from the four stages measured by DPPH and FRAP was similar (8.2 μMET/g). In conclusion, RS influences phenolic and flavonoid contents of 'Ataulfo' mango fruit, which was related with the antioxidant capacity of this fruit.
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1. Introduction

Several clinical and epidemiological studies have demonstrated that fruits and vegetables contain bioactive compounds with antioxidant and antimicrobial activities (Yahia, 2010). These compounds can be of different chemical classes such as phenolic compounds, carotenoids and vitamins (Gonzalez-Aguilar et al., 2008). Mango (*Mangifera indica* L.) fruit can be considered a good source of dietary antioxidants, such as ascorbic acid, carotenoids, and especially phenolic compounds (Ma et al., 2011), which have demonstrated different health-promoting properties, mainly due to their remarkable antioxidant capacity (Kim et al., 2007). Bioactive compounds prevent cardiovascular diseases (Hu, 2003), atherosclerosis, and decrease the risk of some types of cancers, among other health benefits (Yahia, 2010). Thus, regular consumption of mango could provide significant amounts of bioactive compounds with antioxidant activity.

Mango is a popular and economically important tropical fruit throughout the world, due to its excellent eating quality (bright color, sweet taste and luscious flavor) and nutritional composition (vitamins, minerals, fiber, and phytochemicals) (Kim et al., 2009). Global production reached 39 million tons in 2009, followed by banana, pineapple, papaya and avocado (FAOSTAT, 2009). India is the principal mango producer with 35% of the world's production (13.6 million tons), followed by China, Thailand, Indonesia, Mexico and others (FAOSTAT, 2009). However, Mexico is the leading mango-exporting country (41% of the world market), being 'Ataulfo' mango the most important cultivar exported from Mexico to the United States (SAGARPA, 2008).

Recently, it was reported that 'Ataulfo' mango had the highest phenolic content and antioxidant capacity among several mango varieties (Manthey and Perkins-Veazie, 2009). The antioxidant capacity of fruits and vegetables has been correlated to their total phenolic content and composition (Corral-Aguayo et al., 2008). Different factors are reported that affect this antioxidant capacity, such as cultivar, agronomic conditions, post-harvest manipulation and stage of ripeness (Kevers et al., 2007). Although total phenolic compounds have been determined in mango and other tropical fruits, there is a lack of knowledge about the composition and changes of phenolic compounds during maturation and ripening of these fruits.

Various techniques have been developed and used to evaluate the antioxidant capacity of different fruits, and it is suggested to use a combination of at least two of them to estimate the total

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Fig. 1. Four selected ripeness stages (RS) in mango (*Mangifera indica* L., cv. Ataulfo). RS1, representing mango with yellow surface area of 0–10%; RS2, 20–30%; RS3, 70–80% and RS4, 100% yellow color.

antioxidant capacity. The DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) methods are the most commonly used, mainly because of their easy performing, high reproducibility and accuracy (Corral-Aguayo et al., 2008; Ma et al., 2011; Vijaya Kumar Reddy et al., 2010).

The nutritional status of fruit is well correlated with the storage life and resistance against different stresses. The long shelf life of 'Ataulfo' mango compared to other cultivars (Kent, Keitt, Haden and Manila) has been attributed to its high vitamin C content and antioxidant potential (Robles-Sánchez et al., 2009a,b). However, the changes in these parameters during ripening of 'Ataulfo' mango are unknown. Therefore the objective of this work was to evaluate the effect of ripening stage on physiological and quality parameters, phenolic content and composition, and antioxidant capacity of 'Ataulfo' mango fruit.

2. Materials and methods

2.1. Fruit material

Fresh mango fruit (average weight of 200–300 g) (*M. indica* L., cv. Ataulfo) were harvested from a field in Tepic, Nayarit, Mexico, and transported immediately to the laboratory for evaluation. Fruit were selected according to their size, color and appearance discarding fruit with defects and physiological disorders. Afterwards, fruit were sanitized with chlorinated water (200 ppm sodium hypochlorite) for 3 min and left to dry at room temperature (23–26 °C) for about 1 h. Fruit were subjectively selected according to peel surface color and divided in 4 groups of 16 fruits each. Four ripening stages (RS) were established as: RS1, representing mango with yellow surface area of 0–10%; RS2, 20–30%; RS3, 70–80% and RS4, 100% yellow color (Fig. 1). Four mango fruit were taken and the peel was removed with a sharp knife and cut as quickly as possible to obtain the pulp that was cut into small pieces and frozen at −80 °C. After 24 h, the frozen samples were dehydrated in a freeze dryer Labconco Model 1 (Labconco Corp., USA) at −50 °C/0.055 ambar for 36 h, and stored at room temperature in the dark until analyses. The remaining fruit were used for physiological and chemical analysis.

2.2. Physiological and chemical evaluations

Pulp and skin color were longitudinally determined on four points of each flat side of 12 fruit, using a Minolta CR- 300 colorimeter (Konica Minolta Sensing, Inc., USA). The L* value represents the luminosity of the fruit, where O = black and 100 = white. The a* value ranges from the negative (green) to the positive (red) scale while the b* value ranges from negative (blue) to positive (yellow) scale. To know the real color changes of the fruit, a* and b* values were used to calculate the Hue angle (°Hue) value.

Respiration and ethylene production were determined using 4 whole fruit per RS. The mango fruit were placed in sealed plastic containers for 2 h. One milliliter from the headspace was withdrawn using a hypodermic needle, and injected into a Varian Star 3400 CX gas chromatograph (Chromatography system, USA), equipped with a Haysep N column (Chromatography system, USA) of 200 mm in length and internal diameter of 3 mm; 80/100 μm size; with two detectors connected in series; a Thermal Conductivity (TCD) and Flame Ionization (FID) for the quantification of CO_2 and ethylene, respectively. N_2 was utilized as a carrier gas and the temperature conditions were: 50 °C for the column, 70 °C for the injector, 170 °C for the TCD detector and 205 °C for the FID detector. Concentrations of the standards used were 5% O_2, 5% CO_2 and 1 ppm for C_2H_4. To determine the concentration of each gas, the area under the curve was integrated and compared with areas of the known standards.

After measuring respiration of Mango, pulp tissue firmness was measured by the puncture method, using a Chatillon Penetrometer, Model DFM50 (Ametek Inc., USA) with 8 mm diameter flat-head stainless-steel cylindrical probe. Tissue's opposition force against the penetration was registered on 3 points in the equatorial region of the whole piece of fruit with skin removed and results were reported in Newton (N).

The pH and total soluble solids (TSS) contents were evaluated in a 10 g sample of the fruit pulp that were homogenized in 50 mL of distilled water; the mixture was filtered and 50 mL of the filtered mixture were taken to quantify pH, using a Mettler automatic Tritator Model DL21 (Corning Scientific Instruments, USA). TSS was measured directly from the filtered residue, using an Abbe digital refractometer (E-Inginst Electron Corp., USA) and expressed as °Brix.

2.3. Phenolic content and antioxidant evaluation

Freeze-dried mango pulp samples (1 g) were homogenized in 10 mL solution of 80% methanol and 2% formic acid, using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC) at room temperature. The homogenate was sonicated for 30 min in a Bransonic 2210 sonicator (Bransonic Ultrasonic Co., Danbury, CT) and then centrifuged at 9400 × g for 25 min at 4 °C. The supernatant was collected and the precipitate was extracted again with 10 mL of 80% methanol, under the conditions previously described. The two supernatants were mixed, filtered using Whatman filter paper No.1. The final methanolic extract was stored at −25 °C to be used in the determination of total phenolic acids and flavonoids, and for the DPPH and FRAP assays. The extraction process was performed in six replicates per each RS.

Total phenolic acids were determined according to Singleton and Rossi (1965), with some modifications. Results were expressed in mg of gallic acid equivalents (GAE)/100 g of fresh weight (FW).

Total flavonoids were determined with 5% $NaNO_2$, 10% $AlCl_3$ and 1 mol L^{-1} NaOH (Kim et al., 2003). The reaction was placed in a microplate and absorbance was read using an Omega spectrophotometer (BMG Labtech Inc., Germany) with a microplate reader unit, at 510 nm using catechin as standard. The results were expressed as mg of catechin equivalents (CE)/100 g of fresh weight (FW).

DPPH was determined according to the method reported by Brand-Williams et al. (1995) with some modifications. The stock solution was prepared by mixing 2.5 mg of DPPH radical with 100 mL of pure methanol. The solution was adjusted at an absorbance of 1.0 ± 0.02 at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic) was used as a standard and 80% methanol was used as a blank. Samples of 20 μL of the extract (1:10 dilution) were placed in a microplate and 280 μL of DPPH radical were added. The mixture was kept in the dark for 30 min. The absorbance was read using an Omega spectrophotometer (BMG Labtech Inc., Germany) with a microplate reader device, at a wavelength of 490 nm. The inhibition percent was calculated for each sample, which indicates the capacity of the antioxidants to reduce

Table 1
Changes in total soluble solids (TSS), pH, firmness and color parameters (°Hue and L*) in mango (*Mangifera indica* L., cv. Ataulfo) at four ripening stages.

Ripeness stages	TSS (°Brix)	pH	Firmness (N)	Color	
				Pulp (°Hue)	Peel (L*)
1	14.5*a*	1.3*a*	23.8*a*	102*a*	64*a*
2	17.2*b*	2.6*b*	14.8*b*	99*b*	67*b*
3	18.4*c*	3.2*c*	12.4*c*	89*c*	72*c*
4	21.6*d*	4.1*d*	11.7*c*	85*c*	75*d*

Mean values in each column followed by a different letter at each ripeness stage are significantly different ($P \leq 0.05$).

the absorbance of the radical after incubation time. Finally, the results were expressed in Trolox equivalents (TE) per 100 g of FW.

FRAP was determined in the sample extracts according to Benzie and Strain (1999). The method is based on the ability of the sample to reduce Fe^{3+} to Fe^{2+} ions. In the presence of TPTZ, the Fe^{2+}–TPTZ complex exhibits blue color at 593 nm. Briefly, 280 μL of FRAP reagent is added to 20 μL of the extract (1:10 dilution). The mixture was kept for 30 min in the dark. The absorbance was read using an Omega spectrophotometer (BMG Labtech Inc., Germany) with a microplate reader device, at a wavelength of 630 nm. Calibration curve was prepared using an aqueous solution of Trolox as standard. Results were expressed in Trolox equivalents (TE) per 100 g of FW.

Relative total phenolic antioxidant capacity (RTPAOC) was calculated according to Tabart et al. (2009), where the RTPAOC represents the average between DPPH and FRAP values of each RS, calculated in Trolox equivalents.

2.4. Statistical analysis

Results were expressed as means ± SD. Data were statistically analyzed by one-way ANOVA procedure, and the Tukey–Kramer multiple comparison tests were used. The statistical software used was Statgraphics Plus for Windows® v. 5.0. Four replicates were used for each experiment.

3. Results

3.1. Physiological and chemical evaluations

As expected in a climacteric fruit, a lower CO_2 production was observed in green fruit (RS1) compared to more mature stages (RS2, RS3 and RS4). The respiration rate was 3-fold higher (122.2 mL CO_2/(kg h) on average) during stages RS2 to RS4 compared to RS1 (37.5 mL CO_2/(kg h)) (Fig. 1A). Maximum production of ethylene was observed in fruit from RS3 (0.74 μL C_2H_4/(kg h)) (Fig. 2B), which also had the maximum production of CO_2 as expected for fruits in the climacteric peak.

Pulp firmness decreased during ripening. In this study, fruit firmness values ranged from 24.3 N (RS1) to 11.2 N (RS4), pH from 1.3 (RS1) to 4.1 (RS4) and TSS increased from 6.5 °Brix in RS1 to 21.3 °Brix in RS4 (Table 1).

The external color of 'Ataulfo' mango fruit changed from green to yellow. As for the different tissues, peel color is reported as lightness in peel, and pulp color in °Hue angle. A slight decrease in color (°Hue) was observed in mango pulp from 104 to 85, which was not statistically different ($P > 0.05$) between fruit of RS3 and RS4.

3.2. Biochemical evaluations

Total content of phenolic content and flavonoids were evaluated and correlated with the antioxidant capacity. Total phenolic contents increased from RS1 to RS3 fruit and then decreased in RS4 (Fig. 3A). On the other hand, the flavonoids content in RS1 fruit was about 8.5 mg QE per 100 g FW (Fig. 3B), without significant differences with respect to RS2, RS3 and RS4 fruit.

Fig. 4A shows the antioxidant capacity (TE/100 mg FW) present in 'Ataulfo' mango fruit at different ripeness stages evaluated by the DPPH assay. The lowest antioxidant capacity was observed, as expected, in RS1 fruit (211.93 mg TE/100 g FW) and the highest in RS3 fruit (313.4 mg TE/100 g FW), however these fruit had a similar antioxidant capacity as RS2 fruit.

Fig. 4B shows the antioxidant capacity measured by FRAP assay (TE/100 mg FW) of 'Ataulfo' mangoes at different ripeness stages. The antioxidant activity barely changed in fruit of the four ripeness stages with an average value of 211.93 mg TE/100 g FW.

We calculated a relative total phenolic antioxidant capacity (RTPAOC) of the 'Ataulfo' mangoes from the average between DPPH and FRAP values both calculated in Trolox equivalents. RTPAOC results are showed in Fig. 4C. A high antioxidant capacity was observed in RS1 fruit, which increased significantly in a similar extent in RS2 and RS3 without significant differences among them (232.63 mg TE/100 g FW on average), and the lowest was observed in RS4 fruit (196.24 mg TE/100 g FW). These results positively

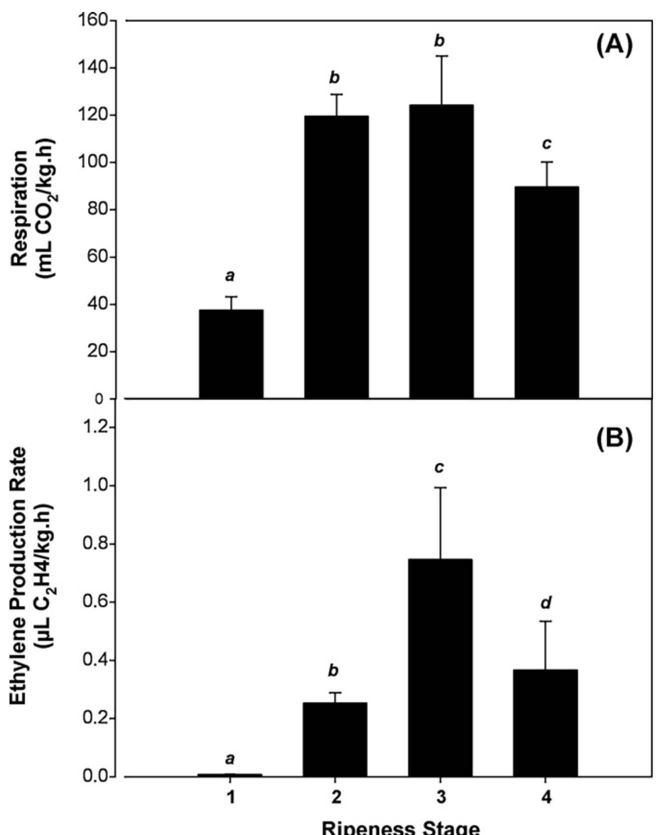

Fig. 2. Respiration (A) and ethylene production (B) of mango (*Mangifera indica* L., cv. Ataulfo) at four ripening stages. Data are means of at least three determinations. Mean values in each bar followed by a different letter at each ripeness stage are significantly different ($P \leq 0.05$).

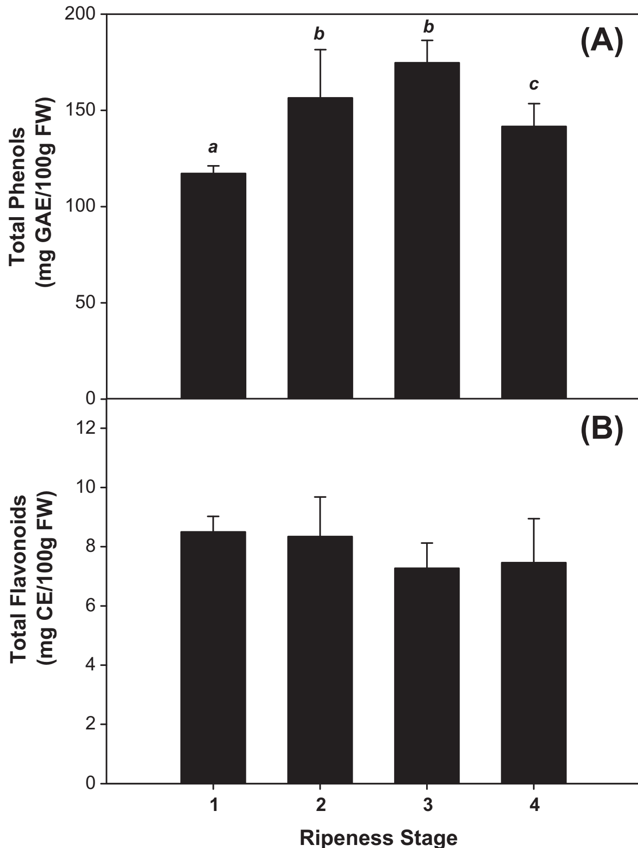

Fig. 3. Total phenolic acids (A) and flavonoids (B) in mango (*Mangifera indica* L., cv. Ataulfo) at four ripening stages. CE (catechin equivalents), FW (fresh weight). Data are means of at least three determinations and error bars indicating the standard deviation. Mean values in each bar followed by a different letter at each ripeness stage are significantly different ($P \leq 0.05$). Flavonoids results showed no significant differences ($P > 0.05$).

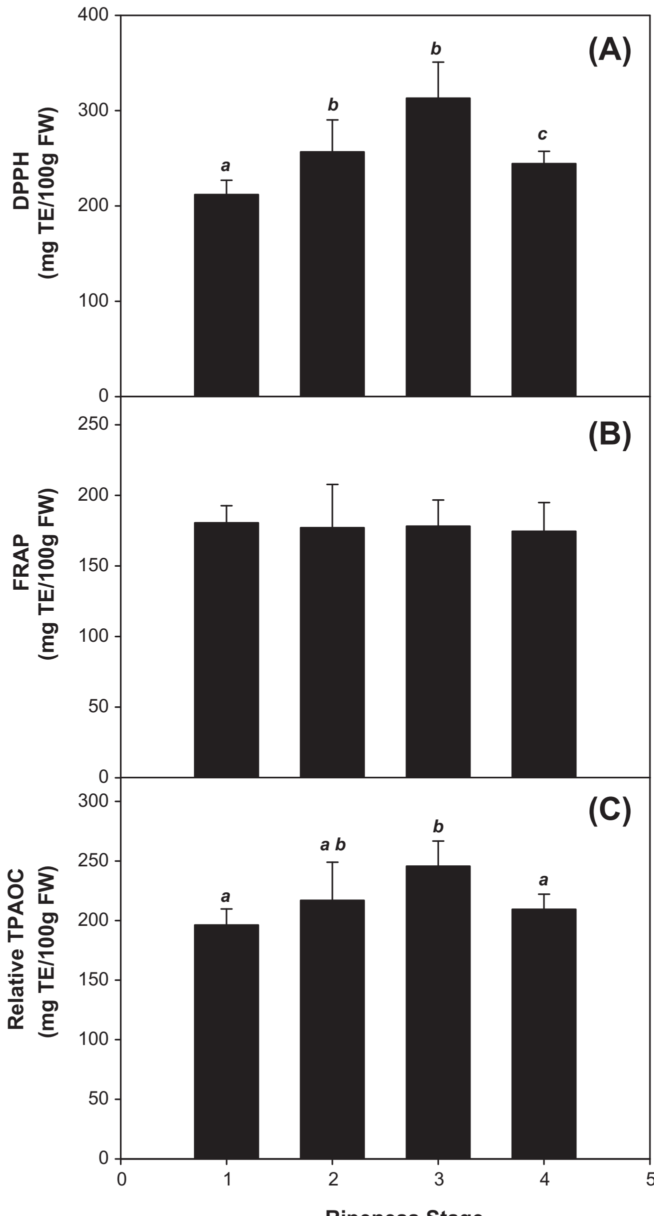

Fig. 4. Antioxidant capacity measured by DPPH (A), FRAP (B) and as relative total phenolic antioxidant capacity (RTPAOC) (C) measured as the average of addition between the results of DPPH and FRAP in mango (*Mangifera indica* L., cv. Ataulfo) at four ripening stages. TE (Trolox equivalents), FW (fresh weight). Data are means of at least three determinations. Mean values in each bar followed by a different letter at each ripeness stage are significantly different ($P \leq 0.05$). FRAP results showed no significant differences ($P > 0.05$).

correlate (Table 2) with total phenolic content (Fig. 3A), and also with the behavior observed in respiration rate (Fig. 2A).

4. Discussion

The respiration rate values were similar to those reported by Montalvo et al. (2007) for 'Ataulfo' mango with average value of 109.2 mL CO_2/(kg h) at 25 °C. Several studies on tropical fruits like mango and papaya have noted that the advance in the stage of ripeness and/or the higher in storage temperature, the higher the respiration rate, with a decreasing pattern in the later stages of ripeness and at very high temperatures (Rivera-López et al., 2005). Ripening is accompanied by an increase in fruit respiration. Climacteric fruits such as mango are characterized by a sudden increase in their respiration rate and ethylene biosynthesis patterns during the ripening process (White, 2002).

Table 2
Positive Pearson correlation coefficients' (r) and probability level (P) of linear regression among different antioxidants and physiological evaluations.

Evaluations correlated	*r*	*P*
Respiration–ethylene production	0.349	0.0026
Total phenols–respiration	0.628	0.0001
DPPH–total phenols	0.781	0.0001
FRAP–total flavonoids	0.412	0.0003
RTPAOC–respiration	0.4288	0.0001

DPPH: 2,2′-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; RTPAOC: relative total phenolic antioxidant capacity.

Ethylene plays an important role in maturation and ripening of climacteric fruits where an increased biosynthesis of this hormone is observed. In accordance to Montalvo et al. (2007) the ethylene production observed in "Ataulfo" mango of different RS, increased before reaching the climacteric peak and decreased afterwards.

One of the most important factors that affect firmness is the modification of cell walls of fruit cells and their degradation by pectin methyl esterase (PME), polygalacturonase (PG) and β-galactosidase enzymes that commonly increase in activity during the last stages of ripening (Gonzalez-Aguilar et al., 2008). Our results were in similar patterns in firmness loss as those reported by Robles-Sánchez et al. (2009b) in 'Ataulfo' mango fruit.

The increase in TSS content during ripening is attributed to the accumulation of free sugars from the hydrolysis of starch by the

action of amylases that are normally ethylene-dependent (White, 2002). Also, according to Jacobi et al. (2000) and Tovar et al. (2001), acidity decreases as mangoes ripen, mainly since citric, ascorbic and malic acids are used as substrates during respiration. In general, our TSS values tended to increase, while acidity tended to decrease with fruit ripening.

The color change of mango is a reliable parameter to determine the extent of fruit ripening (Ninio et al., 2003; Ornelas-Paz et al., 2008). Our results are similar to those reported by Ornelas-Paz et al. (2008) and Robles-Sánchez et al. (2009b) in 'Manila' and 'Ataulfo' mangoes, respectively. Carotenoids biosynthesis increase in most mango varieties and is associated with the climacteric increase in respiration that is initiated by the action of ethylene (Saltveit, 1999).

The phenolic content of mango fruit is similar to that reported by Robles-Sánchez et al. (2009b) in 'Ataulfo' mango with a content of 120 mg GAE/100 g FW. An increase or no change of total phenolic content during ripening have been reported in recent studies (Kim et al., 2009; Robles-Sánchez et al., 2009b). Similarly, the antioxidant capacity was practically unchanged, as observed in total soluble phenolics. However, in these studies the total phenols tended to decrease with fruit ripening and have been related to fruit senescence, which coincide with our results of respiration mentioned above (Fig. 1A). Since that ascorbic acid and other organic acids react with the Folin–Ciocalteu reagent in the phenol assay, the decrease in the phenolic content could be related to the citric acid losses due to the fact that ascorbic and malic acids are used as respiratory substrates. Ascorbic acid is the main biologically active form of vitamin C, and contrary to other organic acids, vitamin C is quite unstable (Robles-Sánchez et al., 2009b).

On the other hand, the gradual increase in total soluble phenolics was reported in mangoes, as starch was converted to simple sugars by amylase activity during storage (Gil et al., 2000). It has been reported that gallic acid and gallotannins are the main phenolic compounds found in mango fruit (Kim et al., 2007; Masibo and He, 2008). Kim et al. (2009) observed during 4 days storage that gallic acid and gallotannins practically remain unaltered. This suggests that these compounds probably maintain the phenolic content in the first ripeness stages. Soong and Barlow (2006) suggested that the lack of change in gallic acid and increased gallotannin concentration may be explained by enzyme-induced hydrolysis of high molecular weight tannins. Berardini et al. (2004) previously showed that mango contain numerous high molecular weight gallotannins that can be broken down into smaller gallotannins. It is possible that biosynthesis of gallotannins occurred via galloyltransferases present in mangoes in the phenilpronoids pathway (Jaakola et al., 2002). However, there is a lack of information relating to gallic acid and gallotannin stability in fresh mangoes, and the postharvest physiology of these two major phenolic compounds is still unclear.

With regards to flavonoids, our results are similar to those reported by Robles-Sánchez et al. (2009b) where no changes were observed in 'Ataulfo' mangoes, during 15 days at 5 °C, with values of about 17.5 mg quercetin equivalents/100 g (FW). In this study it appears that phenolics are responsible for the high antioxidant capacity.

It has been observed that flavonoid contents correlate with the reduction of deteriorative reactions (Crozier et al., 2000; Ross and Kasum, 2002). The higher flavonoid content present in 'Ataulfo' mangoes could be associated with their long shelf life, as it has been reported in other important products (Tomás-Barberán and Espín, 2001). From our results it seems that ripening does not affect the content of mango flavonoids since they were similar in fruits of the four RS. Future studies should be focused on the evaluation of changes and on the preservation of these compounds in mango and other tropical fruits, such as specific molecules as quercetin and catechin being the main flavonoids present in mango (Shivashankara et al., 2004). These flavonoids are potent antioxidants with beneficial health effects (Pietta, 2000).

There are many methods used to determine total antioxidant capacity, and it is important to point out that all of them have some limitations (Robles-Sánchez et al., 2009a). It has been observed that some antioxidant assays give different trends. For that reason multiple methods to generate an 'antioxidant profile' are needed (Gayosso-García Sancho et al., 2010; Robles-Sánchez et al., 2009a; Sanchez-Moreno, 2002). According to previous experience (Gorinstein et al., 2006; Karadag et al., 2009) the best combination of the antioxidant tests for different fruits was TEAC or FRAP and DPPH, and the last two were used in this study.

The DPPH method has been recently used by Ribeiro et al. (2008) and Ma et al. (2011), they observed that antioxidant capacity measured as RSA% was higher in 'Ataulfo' mango, compared with other cultivars such 'Manila', 'Kent', 'Palmer', 'Tommy Atkins' and others, attributing these values to the considerable amount of phenolic compounds present in 'Ataulfo' mango fruit. On the other hand, DPPH results correlate with total phenolic content (Table 2). This was expected and in agreement with other studies (Gayosso-García Sancho et al., 2010; Ma et al., 2011; Ribeiro et al., 2008; Robles-Sánchez et al., 2009a; Sanchez-Moreno, 2002). The DPPH results are used to confirm the results obtained in total phenolic content (Karadag et al., 2009; Tabart et al., 2009). This was confirmed in our study.

FRAP assay has been considered a good method to evaluate the antioxidant capacity of different tropical and exotic fruits including several mango cultivars (Guo et al., 2003; Luximon-Ramma et al., 2003; Nilsson et al., 2005; Saura-Calixto and Goñi, 2006; Vasco et al., 2008). However, FRAP has some disadvantages like most of the other commonly used methods. Any electron-donating substance even without antioxidant properties with redox potential lower than that of the redox pair Fe(III)/Fe(II) can contribute to the FRAP value and indicate falsely high values (Nilsson et al., 2005). Potential problems take place as the mixture contains other Fe(III) species, which can bind to chelators in the food extract and these complexes are capable of reacting with the antioxidants. Results show that, similar to TEAC, there is no relationship between the FRAP value and the number of electrons that an antioxidant can donate (MacDonald-Wicks et al., 2006). The FRAP values for ascorbic acid, α-tocopherol, and uric acid were identical, but they all showed different FRAP values. So, probably other compounds or the individual contribution of phenolic compounds and their proportions present in the different ripeness stages maintain the FRAP values unaffected and constant.

In general, phenolic compounds can act as antioxidants and their antioxidant activity or capacity is determined according to their chemical structure or due to their interaction with other antioxidants. To date, no standardized assay or universal method is available to estimate the total antioxidant activity in fruits. The nature of the food sample, the antioxidant components, the reaction mechanism of the oxidants, and the measurement of end point oxidation make this task difficult (Sanchez-Moreno, 2002).

As recognized by several authors (Frankel and Meyer, 2000; Heo et al., 2007; Huang et al., 2005; Robles-Sánchez et al., 2009b; Tabart et al., 2009) no single method is adequate for evaluating the antioxidant capacity of foods, since different methods can yield widely diverging results. However, Tabart et al. (2009) proposed to standardize reports on antioxidant capacity, using an average of the results obtained by the several methods like DPPH, oxygen radical antioxidant capacity (ORAC), hemolysis, and electron spin resonance (ESR) methods with common standard such as Trolox among methods. This supported that a simple mathematical mean is not adequate, because two of the four methods gave much higher values because of the poor performance of Trolox in these assays. This would give those assays undue preponderance in the mean.

In our study, we followed the same procedure with our data. We determined the average values between DPPH and FRAP values, since both were calculated in Trolox equivalents, and finally we obtained a RTPAOC for 'Ataulfo' mangoes.

This suggests that the physiological and ripening process in mangoes directly affects the presence of phenolic content and their antioxidant activity. Our hypothesis establishes that in climacteric fruits such as mango, the cellular activity is remarkably high during ripening where most of the biomolecules are being metabolized (Payasi et al., 2009; Tovar et al., 2001). In this process, the fruits need important amount of energy to support all the physiological pathways in the cell (Vishwas et al., 2010) The generation of energy by the respiratory system may generate free radicals and oxygen reactive species at the end of the electron transporting chain (Masibo and He, 2008). The mango fruits may need to activate antioxidants defense mechanisms to avoid oxidative stress, thus activating the synthesis of phenolic compounds by the phenilpropanoid pathway. However, the study of related enzymes responsible for phenolic biosynthesis during ripening of 'Ataulfo' mango and also expression of the genes encoding these enzymes are being analyzed in our group of work and may be useful to understand and explain the metabolic changes of these compounds during ripening.

In summary, phenolic compounds have different antioxidant capacity, depending on their structure, number of hydroxyl groups, as well as the matrix where they are embedded (Heo et al., 2007). Determination of the antioxidant capacity in fruits should take into account the overall concentration and composition of diverse antioxidants, because the total antioxidant capacity is in function of the combined activities of diverse antioxidants. Thus, in further studies it may be convenient to evaluate individual phenolic content and their relative individual contribution to the antioxidant capacity of 'Ataulfo' mango, in order to understand the authentic changes that occur during ripening and possible positive effects as part of a healthy diet. A follow-up of ripening stages is useful to understand the health benefits of mango in the diet.

5. Conclusions

Fruit physiological and ripening processes influence the content and antioxidant activity of phenolic compounds in 'Ataulfo' mango fruit. Flavonoid content was not affected by ripening. Additional studies of phenolic biosynthesis and analysis of their biosynthesis enzymes expression are recommended to enrich the understanding of their metabolism.

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II

IDENTIFICATION AND QUANTIFICATION OF MAJOR PHENOLIC COMPOUNDS FROM MANGO (*Mangifera indica*, cv. Ataulfo) FRUIT BY HPLC–DAD–MS/MS-ESI AND THEIR INDIVIDUAL CONTRIBUTION TO THE ANTIOXIDANT ACTIVITY DURING RIPENING

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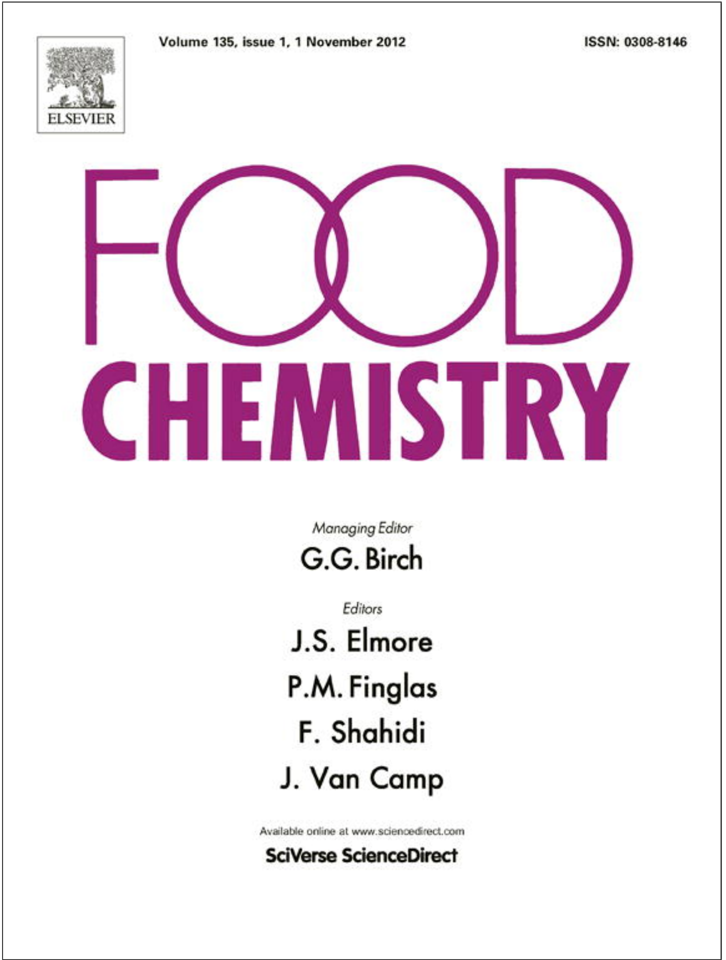

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Identification and quantification of major phenolic compounds from mango (*Mangifera indica*, cv. Ataulfo) fruit by HPLC–DAD–MS/MS-ESI and their individual contribution to the antioxidant activity during ripening

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ABSTRACT

Mango (*Mangifera indica* L.) is an economically important fruit throughout the world. 'Ataulfo' mango, a leading cultivar in Mexico, has the highest content of phenolic compounds among several commercial varieties of mango. However, the individual identification and antioxidant contribution of these phenols during ripening of mango fruit is unknown. Qualitative and quantitative analysis of the major phenolic compounds found in 'Ataulfo' mango fruit pulp was conducted in four stages of ripeness, using high-performance liquid chromatography coupled to mass spectrometry. The antioxidant contribution of each of the major phenolic compounds was calculated. The major compounds identified were chlorogenic acid (28–301 mg/100 g DW), gallic acid (94.6–98.7 mg/100 g DW), vanillic acid (16.9–24.4 mg/100 g DW), and protocatechuic acid (0.48–1.1 mg/100 g DW). The antioxidant contribution of the four phenolic acids increased during ripening. Gallic acid accounted for the highest contribution (39% maximum value), followed by chlorogenic acid (21% maximum value). This could indicate that these phenolic compounds may have an important role in the antioxidant metabolism in 'Ataulfo' mango fruit during ripening, and promoting health benefits to consumers.

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1. Introduction

Several clinical and epidemiological studies have demonstrated that fruits and vegetables contain bioactive compounds with antioxidant activity that promote health benefits (Yahia, 2010). These compounds can be of different chemical classes such as phenolic compounds, carotenoids and vitamins (González-Aguilar, Celis, Sotelo-Mundo, De La Rosa, Rodrigo-Garcia, & Alvarez-Parrilla, 2008). Mango (*Mangifera indica* L.) fruit is considered a good source of dietary antioxidants, such as ascorbic acid, carotenoids, and phenolic compounds (Ma et al., 2011), which have demonstrated different health-promoting properties, mainly due to their remarkable antioxidant capacity (Kim, Brecht, & Talcott, 2007). Bioactive compounds have been reported to prevent cardiovascular diseases (Hu, 2003), atherosclerosis, and decrease the risk of some types of cancers, among other health benefits (Yahia, 2010). Thus, regular consumption of mango could provide significant amounts of bioactive compounds with antioxidant activity and health benefits.

Mango is a popular and economically important fruit in several parts of the world, due to its excellent sensorial properties (bright colour, sweet taste and luscious flavour) and nutritional composition (vitamins, minerals, fibre, and phytochemicals) (Kim, Lounds-Singleton, & Talcott, 2009). It has been reported that 'Ataulfo' mango had the highest phenolic content and antioxidant capacity amongst several mango varieties (Manthey & Perkins-Veazie, 2009). The antioxidant capacity of fruits and vegetables has been correlated to their total phenolic content and composition (Corral-Aguayo, Yahia, Carrillo-Lopez, & González-Aguilar, 2008). Different factors affect this antioxidant capacity, such as cultivar, agronomic conditions, post-harvest manipulation and stage of ripeness (Ribeiro, Barbosa, Queiroz, Knodler, & Schieber, 2008). Although total phenolic compounds and antioxidant activity have been determined in mango and other fruits, there is a lack of knowledge about the composition and changes of phenolic compounds, and moreover, their antioxidant capacity during ripening of these fruits.

Phenolic compounds have different antioxidant capacity, depending on their structure, number of hydroxyl groups and their distribution in the structure (Heo, Kim, Chung, & Kim, 2007). Determination of the antioxidant activity in fruits should take into account the overall concentrations and compositions of diverse phenolic compounds, at least the most abundant. The antioxidant activity in 'Ataulfo' mango is due to the individual and also combined activities of those antioxidants. Thus, it is convenient to

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evaluate individual phenolic contribution to the 'Ataulfo' mango antioxidant activity, in order to understand the authentic changes that occur during ripening. The objectives of this work were to identify, quantify and evaluate the individual antioxidant contribution of the major phenolic compounds found in 'Ataulfo' mango pulp at different ripening stages.

2. Materials and methods

2.1. Fruit material

Fresh mango fruit (average weight of 250 g) (*M. indica* L. cv. Ataulfo) were harvested from a field in Tepic, Nayarit, Mexico, and transported immediately to the laboratory for evaluation. Fruit were selected according to their size, colour and appearance discarding fruit with defects and physiological disorders. Afterwards, fruit were sanitized with chlorinated water (200 ppm sodium hypochlorite) for 3 min and left to dry at room temperature (23–26 °C) for about 1 h. Fruit were subjectively selected according to peel surface colour and divided in 4 groups of 16 fruits each. Four ripening stages (RS) were established as: RS1, representing mango with yellow surface area of 0–10%; RS2, 11–40%; RS3, 41–70% and RS4, 71–100%. Four mango fruit were taken and the peel was removed with a sharp knife and cut as quickly as possible to obtain the pulp that was cut into small pieces and frozen at −80 °C. After 24 h, the frozen samples were dehydrated in a freeze drier Labconco Model 1 (Labconco Corp., Kansa City, USA) at −50 °C/0.055 ambar for 36 h, and stored at room temperature in the dark until analyses.

2.2. Phenolic extracts and antioxidant evaluation

Freeze-dried mango pulp samples (1 g) were homogenized in 10 mL solution of 80% methanol and 2% formic acid, using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC) at room temperature. The homogenate was sonicated for 30 min in a Bransonic 2210 sonicator (Bransonic Ultrasonic Co., Danbury, CT) and then centrifuged at 9400*g* for 25 min at 4 °C. The supernatant was collected and the precipitate was extracted again with 10 mL of 80% methanol, under the conditions previously described. The two supernatants were mixed, and filtered using Whatman filter paper No. 1. The final methanolic extract was stored at −25 °C to be used in the determination of relative total phenolic antioxidant capacity (RTPAOC) using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) assays. Also, total phenols were determined for each RS by the method reported by Singleton and Rossi (1965), and compared to the individual content of phenolic acids.

The DPPH assay was conducted according to the method reported by Brand-Williams, Cuvelier, and Berset (1995) with some modifications. The stock solution was prepared by mixing 2.5 mg of DPPH radical with 100 mL of pure methanol. The solution was adjusted at an absorbance of 1.0 ± 0.02 at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic) was used as a standard and 80% methanol was used as a blank. Samples of 20 μL of the extract (1:10 dilution) were placed in a microplate and 280 μL of DPPH radical were added. The mixture was kept in the dark for 30 min. The absorbance was read using an Omega spectrophotometer (BMG Labtech Inc., Deckenpfronn, Germany) with a microplate reader device, at a wavelength of 490 nm. The inhibition percent was calculated for each sample, which indicates the capacity of the antioxidants to reduce the absorbance of the radical after incubation time. Calibration curve was prepared using an aqueous solution of Trolox as standard.

FRAP was determined in the sample extracts according to Benzie and Strain (1999). The method is based on the ability of the sample to reduce Fe^{3+} to Fe^{2+} ions. In the presence of TPTZ, the Fe^{2+}-TPTZ complex exhibits blue colour at 593 nm. Briefly, 280 μL of FRAP reagent is added to 20 μL of the extract (1:10 dilution). The mixture was kept for 30 min in the dark. The absorbance was read using an Omega spectrophotometer (BMG Labtech Inc., Deckenpfronn, Germany) with a microplate reader device, at a wavelength of 630 nm. Calibration curve was prepared using an aqueous solution of Trolox as standard.

RTPAOC was calculated according to Tabart, Kevers, Pincemail, Defraigne, and Dommes (2009), where the RTPAOC represents the average between DPPH and FRAP values of each RS, calculated both in equal standard as Trolox equivalents.

2.3. Identification and quantification of phenolic acids

2.3.1. Preparation of extracts

Preparation and identification of phenolic acids were determined according to a modified method described by Ferreres et al. (2008). Mango pulp freeze-dried samples (0.5 g) were homogenized in 20 mL of 80% methanol, using an Ultra Turrax®T25 basic homogenizer (IKA Works, Willmington, NC), sonicated for 30 min at 30 °C in a 2510 model ultrasonic bath (Branson, Whetersfield, CT), centrifuged at 12,000*g* for 15 min at 5 °C in a Hermle centrifuge model Z323 K (Labortechnik Technologies, Wehingen, Germany), and then filtered through number 1 Whatman paper. For acidic hydrolysis 2 mL of 2.4 M HCl was added to 2 mL of phenolic extracts and left for 4 h in the dark at 80 °C. After incubation, extracts were filtered through nylon membrane of 0.45 μm of pore size (Millipore Corp., Bedford, MA) and directly injected and analyzed by HPLC/UV–DAD/ESI-MS system.

2.3.2. High-performance liquid chromatography (HPLC)

HPLC analysis was performed according to Rivera-Pastrana, Yahia, and González-Aguilar (2010) and Gayosso-Garcia Sancho, Yahia, and González-Aguilar (2010) Samples containing phenols were injected automatically into an HP 1100 series HPLC system (Hewlett–Packard, Palo Alto, USA) equipped with a diode array detector (DAD). Absorption spectra for the main peaks were recorded at 280 and 320 nm. The HPLC system was equipped with an Xterra RP_{18} reverse phase column (4.6 mm × 250 mm) with a spherical particle size of 5 μm, which was kept at 25 °C. The mobile phase was composed of 1% formic acid (A) and acetonitrile (B), and the elution gradient was 2–100% (B) in 40 min at a flow rate of 0.5 mL/min and 25 °C. The injection volume was 20 μL.

2.3.3. High-performance liquid chromatography–mass spectrometry (HPLC–ESI-MS) analysis

Mass spectra of the main phenolic acids were obtained using the chromatographic system described above but with a 6210 model time-of-flight (TOF) mass spectrometer (Agilent Technologies Inc., Palo Alto, CA) equipped with an electrospray ionization (ESI) source operating at the negative ionization mode and MassHunter manager software (Version A.02.01). High-purity nitrogen (99.999%) was used as nebulizing (45 psig) and drying gas (11.0 L/min); gas and vapourizer temperature was 350 °C; and the corona, capillary, and fragmentor voltages were 4 μA, 4 kV, and 220, respectively. Phenolic compounds were identified by comparing their retention time and UV–Vis data with those obtained with reference standards as well as co-chromatography with added standards and using their mass spectra (m/z 50–800). Quantitative data for phenolics were obtained by calibration curves constructed with known standards.

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2.4. Individual contribution of phenolic compounds to total phenolic antioxidant activity

2.4.1. Individual antioxidant capacity

Pure commercial standards of gallic, chlorogenic, protocateichuic and vanillic acid were prepared in 80% methanol at 1 mM concentration. Antioxidant capacity was determined by DPPH, and reported as percent of radical scavenge capacity (RSA).

2.4.2. Determination of antioxidant contribution

Phenolic compounds preparations were done according to individual phenolic acids concentrations in the methanolic extract from the four RS (Table 1). Pure commercial standards were used for each phenolic preparation. The individual phenol antioxidant contribution (IPAOC) determination was performed for each phenolic acid preparation according to Table 1 using the DPPH assay as described above. The individual contribution to the RTPAOC at each RS was calculated as follow:

$$\% \text{ of Contribution RTPAOC} = \frac{(\text{IPAOC})\ (100)}{\text{RTPAOC}}$$

where RTPAOC, total phenolic antioxidant activity at each ripeness stage; IPAOC, individual phenol antioxidant capacity at particular concentration in the ripeness stage.

2.5. Statistical analysis

Results were expressed as means ± SD. Data were statistically analyzed by one-way ANOVA procedure, and the Tukey–Kramer multiple comparison test was used. Standard deviation and variance coefficient between data groups were used to determine significant differences between them at $P \leqslant 0.05$ using the statistical software Statgraphics Plus for Windows® v. 5.0. Four replicates were used for each experiment.

3. Results and discussion

The major phenolic compounds identified in the four RS were the phenolic acids gallic, chlorogenic, protocatechuic and vanillic (Fig. 1), and some organic acid derivatives (Fig. 1, Table 2). The four RS of 'Ataulfo' mango showed almost identical phenolic profile. According to several authors (Kim et al., 2007, 2009; Robles-Sánchez, Rojas-Graü, Odriozola-Serrano, González-Aguilar, & Martín-Belloso, 2008) phenolic acids are predominant compounds in mango pulp.

Gallic acid was identified as a [M−H]-deprotonated molecule (m/z 169) with an UV spectrum (λ_{max} = 220, 275 nm) in a RT of 15.5 min and yielded ion fragments at m/z 125, 126 and 170 (Table 2). Protocatechuic acid was identified according to its UV spectrum (λ_{max} = 230, 266 nm) as a [M−H]-deprotonated molecule (m/z 153), with a RT of 18.85 min, and yielded ion fragments at m/z 108 and 155. Chlorogenic acid was identified as a [M−H]-deprotonated molecule (m/z 356) with an UV spectrum (λ_{max} = 286, 240 nm) in a RT of 20.05 min. Vanillic acid was identified as a [M−H]-deprotonated molecule (m/z 168), with a RT of 20.75 min, and yielded ion fragments at m/z 124 and 313.

The identified phenolic compounds coincides with those reported by Masibo and He (2008). Also, profile of phenolic compounds but not concentration coincides with reports on the identification of phenolics in 'Ataulfo' (Robles-Sánchez et al., 2009), 'Kent' (Robles-Sánchez et al., 2008) and 'Tommy Atkins' (Kim et al., 2009) mango pulp. Phenolic compounds have been reported to have important antiradical, antimutagenic, and anticarcinogen properties and protect plants from UV radiation (Masibo & He, 2008).

Recent *in vitro* studies on gallic acid have shown strong anticancer efficacy against human prostate cancer cells (Ji et al., 2009; Saxena et al., 2008). Raina, Rajamanickam, Deep, Singh, Agarwal, and Agarwal (2008) reported *in vivo* chemopreventive efficacy against prostate cancer in mice orally administrated with gallic acid. Chlorogenic acid, formed by esterification of caffeic and quinic acids, is one of the most abundant polyphenol in the human diet (Lafay, Morand, Manach, Besson, & Scalbert, 2006). According to dos

Table 1
Concentrations of major phenolic compounds in 'Ataulfo' mango hydrophilic extract from different ripeness stages.

Phenolic Compound	Structure	Ripeness stage 1 (Concentration µg/mL)	2	3	4
Gallic acid	HO, HO, HO, OH, O	62	50	43	57
Protocatechuic acid	HO, HO, OH, O	0.32	0.34	0.4	0.61
Chlorogenic acid	HO, HO, O, O, HO, O, OH, OH, OH	18	67	97	177
Vanillic acid	OH, HO, O, O, CH3	11	9.8	9.3	14

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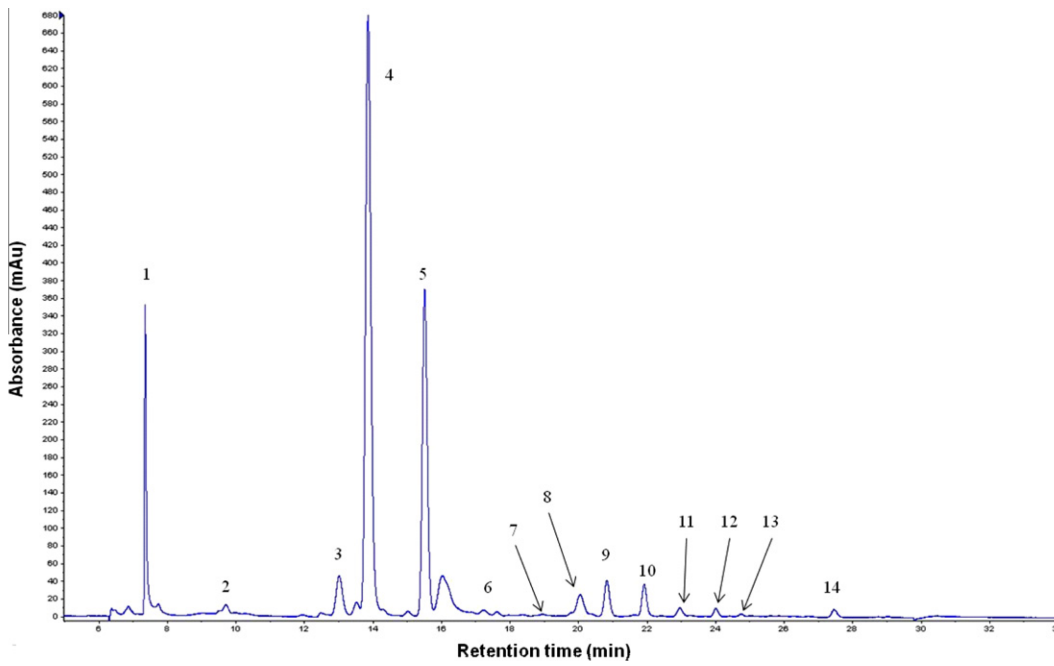

Fig. 1. General HPLC–MS chromatogram profile of 'Ataulfo' mango pulp. Major phenols quantified were: (5) gallic acid, (7) chlorogenic acid, (8), protocatechuic acid and (9) vanillic acid.

Table 2
Phenolic compounds identified in 'Ataulfo' mango pulp by HPLC–MS.

Peak no.	RT (min)	[M−H] (frag. MS^2 m/z)	UV (nm)	Compound
1	7.35	[173] (376)	242, 213	Shikimic acid-hexamalonate
2	9.7	110, 191, 192, 394	200, 300	Unknown
3	13.1	[457] (439, 662)	205, 300	Epicatechin gallate-hexamalonate
4	13.83	[173] (155, 161, 335)	300,200, 230	Shikimic acid-hexoside
5	15.5	[169] (125, 126, 170)	220, 275	Gallic acid
6	17–17.6	(427, 234, 463, 428)	256	Unknown
7	18.85	[153] (108, 155)	230, 266	Protocatechuic acid
8	20.05	[356] (55, 161, 173, 295, 313)	286, 240	Chlorogenic acid
9	20.75	[168] (124, 313)	228, 277	Vanillic acid
10	21.8	(425, 232, 426, 295)	263, 245	Unknown
11	22.9	(234, 295,423, 173,313)	260, 198	Unknown
12	24	(492, 234, 493, 295)	240, 190	Unknown
13	24.65	(297, 157, 593, 169)	240, 190	Unknown
14	27.5	[355] (173, 193, 216, 337)	240, 190	Ferulic acid-hexoside
Other identified compounds				
	6.1	[429] (217, 285)		Luteolin-hexoside
	9.1	[395] (103, 129, 147)		Cinnamic acid-hexamalonate
	6.87	[327] (179, 208, 224, 386)		Sinapic acid-hexomalonate
	12.1	[490] (155, 163, 327)		*p*-Coumaric acid-hexoside

Santos, Almeida, Lopes, and de Souza (2006) chlorogenic acid presented anti-edematogenic and antinociceptive activities in rats models of carrageenin-induced inflammation and formalin-induced pain, respectively. Also, data obtained from *in vivo* and *in vitro* experiments show that chlorogenic acid mostly presents important antioxidant and anti-carcinogenic activities (Farah, Monteiro, Donangelo, & Lafay, 2008; Lafay & Gil-Izquierdo, 2008). Protocatechuic acid, exhibited an antiproliferative effect on HL-60 cells by inducing apoptosis, which was associated with the phosphorylation and suppression of Bcl-2 protein (Tseng, Kao, Chu, Chou, Lin, & Wang, 2000). Finally, vanillic acid is a common phenolic compound found in Chinese herbs or roots used as medicine (Zheng & Wang, 2001). Some anti-proliferative properties have been associated to vanillic acid from olive oil (Owen, Giacosa, Hull, Haubner, Spiegelhalder, & Bartsch, 2000). In this context, since phenolic acids are natural antioxidants in quenching and neutralizing free radicals (Yahia, 2010), changes of these compounds after harvest and during ripening is an important link to the potential human health benefits of mangoes.

The major phenolic acids quantified by HPLC–DAD in mango pulp were chlorogenic, gallic, vanillic and protocatechuic acids. The content of these phenolic acids were quantified at the four RS (Fig. 2). Chlorogenic acid was the most abundant in 'Ataulfo' mango pulp, followed by gallic acid. The order of phenolic acids from most abundance to the lowest was chlorogenic, gallic, vanillic and protocatechuic acids. Chlorogenic acid had a concentration of 28 mg/100 g DW in RS1, increased to 301 mg/100 g DW in RS4. Gallic acid content in RS1 fruit was about 94.6 mg/100 g DW, without significant differences with respect to RS2 and RS3, but in RS4 reached 98.7 mg/100 g DW. The same pattern was observed for

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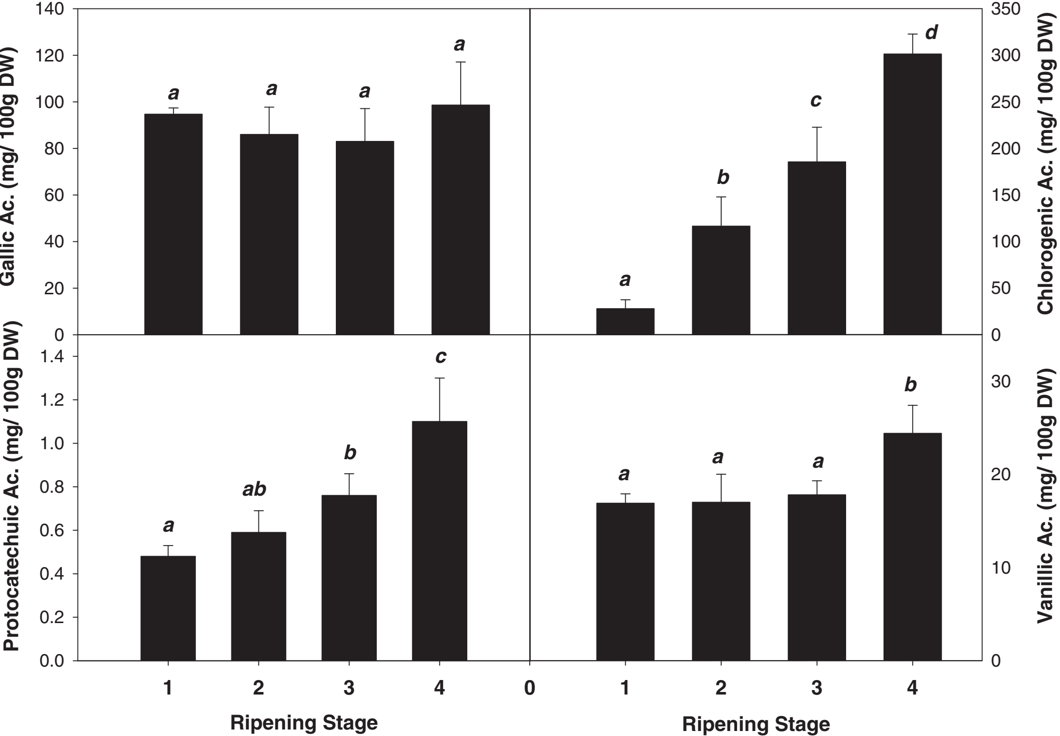

Fig. 2. Changes of the major phenolic compounds at four ripeness stages in 'Ataulfo' mango pulp. Different letter at each ripeness stage indicate significant differences ($P \leqslant 0.05$).

vanillic acid, which had a concentration of 16.9 mg/100 g DW and reaching 24.4 mg/100 g DW in RS4. Finally, protocatechuic acid had a concentration of 0.48 mg/100 g DW in RS1, and increased to 1.1 mg/100 g DW in RS4. Also, the four phenolic acids accounted for 24% of total phenols in RS1, and their content increased during ripening, reaching a 29.7% and 34% in RS2 and RS3, respectively. At RS4 the four phenolic acids represented the 60% of total phenols (data not shown).

In the case of flavonoids, no clear identification was established by HPLC–MS. The distribution of phenolics and flavonoids in mango fruit varies greatly with the cultivar, maturation, pre and post-harvest treatment applied, among others (Manthey & Perkins-Veazie, 2009). Flavonoid identification is commonly reported in mango peel and in a less extent in the pulp. Peel and pulp present important differences in the content of flavonoids and phenolic acids and other antioxidants (Masibo & He, 2008). Recently, we reported that flavonoid content was low in mango pulp, especially in 'Ataulfo' mango (Palafox-Carlos, Yahia, Islas-Osuna, Gutierrez-Martinez, Robles-Sánchez, & González-Aguilar, 2012). Palafox-Carlos et al. (2012), reported that total flavonoids values for 'Ataulfo' pulp was close to 8.5 mg QE per 100 g FW, and when compared with other fruits this content was very low. Also Robles-Sánchez et al. (2009) reported similar flavonoids content for 'Ataulfo' mango pulp. Besides, recent evaluations in our lab on gene *FLS* encoding for flavonol synthase, indicated that the expression of *FLS* in pulp during 'Ataulfo' mango ripening is noticeably low and barely detected (unpublished results). Therefore, it is normal that flavonoids in mango pulp samples were not present in higher concentration as was proved by the HPLC chromatogram profile. However, additional depuration of the HPLC technique would be necessary when the flavonoids identification be of great interest. In this study we focused the discussion around the major phenolic acids found in pulp.

An increase or no change of total or individual phenolic content during mango ripening has been reported in recent studies (Kim et al., 2009; Robles-Sánchez et al., 2009). It has been reported that gallic acid is the main phenolic compound found in mango fruit (Kim et al., 2007; Robles-Sánchez et al., 2008). Kim et al. (2009) observed during 4 days storage that gallic acid practically remain unaltered, which coincided with our findings. On the other hand, Masibo and He (2008) reported that chlorogenic acid is one of the major phenolic compounds found in mango fruit. This suggests that these compounds probably maintain the phenolic content and the antioxidant activity during ripening of mango fruit.

Several epidemiological studies suggest an inverse relationship between consumption of foods rich in phenolic acids and the incidence of various diseases, and chlorogenic and gallic acids may be closely related to those of benefits to consumer. The biosynthesis of chlorogenic and gallic acids occurs via phenylpropanoids pathway (Ferrer, Austin, Stewart, & Noel, 2008), directly connected from phenylalanine via the shikimate pathway (Barone, Calabrese, & Mancuso, 2009). However, there is a lack of information relating to biosynthesis of phenolic compounds in fresh mangoes, and the postharvest physiology of these two major phenols is still unclear.

The individual contribution of phenolic acids to the RTPAOC was evaluated for each RS in 'Ataulfo' mango. Pure standards were used to estimate percentage contribution of the major four phenolic compounds to 'Ataulfo' mango RTPAOC, with the assumption that isolated pure phenolic compounds exhibit similar activity as standards. It is important to mention that their antioxidant contribution would depend not only on the chemical structure of the phenolic acid, but also on their content in the fruit. Thus, it may be convenient to evaluate individual phenol antioxidant contribution, in order to understand the authentic antioxidant changes and phenol participation during fruit ripening.

Fig. 3 shows that at RS1 (green fruit), gallic and vanillic acids contribute the highest IPAOC (28.4% and 3.5%, respectively) followed by chlorogenic and protocateic acids. In total, the four phenolic acids accounted for 33% of RTPAOC. In RS2, the values were similar to those for RS1, but chlorogenic acid significantly

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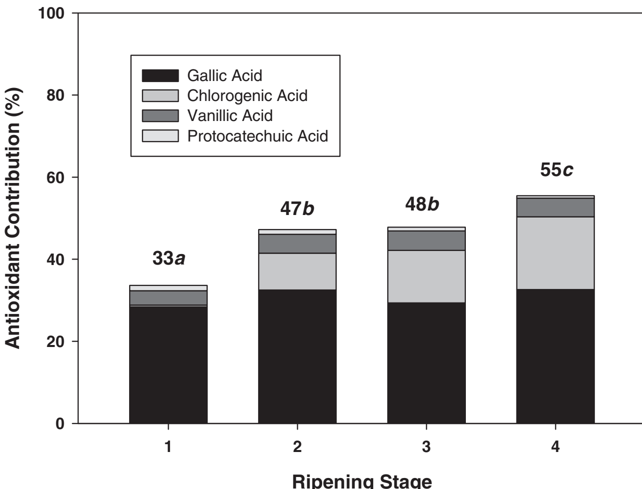

Fig. 3. Individual antioxidant contribution of the major phenolic compounds to the total phenolic antioxidant activity in 'Ataulfo' mango pulp at different ripeness stages. Different letter at each ripeness stage indicate significant differences ($P \leqslant 0.05$).

increased its contribution (8.9%), and the four accounted for 47% of RTPAOC. At RS3, the results showed no significant differences with those of RS2, and the four phenolic acids accounted for 48% of RTPAOC. Finally, at RS4 (yellow fruit), the four phenolic accounted for 55% of RTPAOC. These results indicate that the consumption of ripened mango (RS4) is better due to their higher antioxidant contribution and content of their major phenolic acids, which may contribute to improved human health. Our results agree with those of Reddivari, Hale, and Miller (2007), where gallic acid and chlorogenic acid showed the highest contribution to the antioxidant activity in several varieties of potatoes. It is important to mention that the rest of the antioxidant contribution may be associated to the other diverse phenolic compounds present in the mango pulp, and also to ascorbic acid.

Regarding the different antioxidant contribution values of the four phenolic acids, it has been reported that the phenolic compounds may have different antioxidant potential, depending on their structure conformation, number of hydroxyl groups and their distribution in the structure (Heo et al., 2007). The individual antioxidant capacity of the major phenolic compounds in 'Ataulfo' mangos was determined in order to estimate the antioxidant potential of each phenolic acid. Fig. 4 shows the antioxidant capacity of the four phenolic compounds at equal 1 mM concentration. Gallic acid showed the highest antioxidant capacity with 70% RSA, followed by protocatechuic acid and chlorogenic acid, showing 50% and 42% RSA, respectively. Vanillic acid exhibited the lowest value, showing 15% RSA. Our results coincided with those reported by Rice-Evans, Miller, and Paganga (1996), where gallic acid exhibited the highest antioxidant capacity, and vanillic acid the lowest among several phenolic compounds.

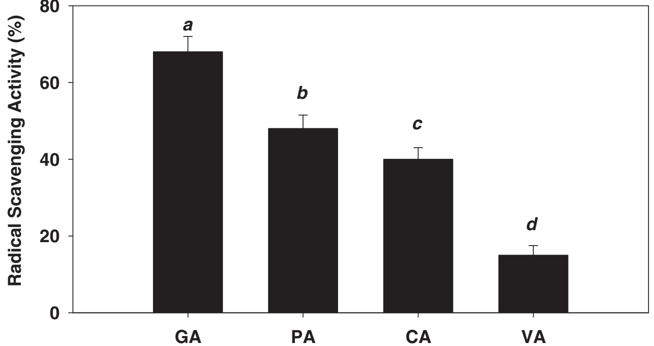

Fig. 4. Individual antioxidant capacity of gallic acid (GA), protocatechuic acid (PA), chlorogenic acid (CA) and vanillic acid (VA) at 1 mM concentration. Different letter at each ripeness stage indicate significant differences ($P \leqslant 0.05$).

In particular, phenolic acids are considered efficient hydrogen donors due to their characteristic carboxylic group, which is easily ionized (Leopoldini, Marino, Russo, & Toscano, 2004). Evaluating the individual antioxidant potential of phenolic compounds is a topic that has taken attention in our laboratory in order to understand real potential and biological action of phenolic antioxidants.

Table 1 shows the chemical structure of the four phenolic acids. Gallic acid presents a carboxylic group, but also shows three additional hydroxyl groups available for hydrogen atom donation which could explain its high antioxidant contribution. Protocatechuic acid presents a similar structure to that of gallic acid, but with one hydroxyl group less. In the case of chlorogenic acid, its structure presents six hydroxyl groups. However, its efficiency for hydrogen atom donation is lower than gallic acid. The explanation behind this fact is not clearly understood. Probably, some steric restrictions are involved among their hydroxyl groups (Gonthier, Verny, Besson, Rémésy, & Scalbert, 2003). Finally, vanillic acid structure presents one hydroxyl group apart from its carboxyl group. Besides, this hydroxyl group may have intra molecular interactions with the oxygen of the oxy-methyl group (Kilmartin, Zou, & Waterhouse, 2001), avoiding the hydrogen atom donation. This may explain its low antioxidant capacity in comparison with other phenolic acids.

In general, our results showed that the contribution of the four phenolic acids to the RTPAOC increased during ripening, in particular gallic acid, which showed the major antioxidant contribution in all ripeness stages. This would indicate an important role of phenolic acids in the antioxidant metabolism during ripening of 'Ataulfo' mango. Also, gallic acid may be an important antioxidant for human health.

Ours results suggest that the physiological and ripening process in 'Ataulfo' mango fruit may affect directly the presence of phenolic compounds content and their antioxidant activity. Our hypothesis establishes that in climacteric fruits such as mango, the cellular activity is remarkably high during ripening (Payasi, Mishra, Chaves, & Singh, 2009; Tovar, García, & Mata, 2001). In this process, the fruit needs important amount of energy to support all the physiological pathways in the cell (Bapat, Trivedi, Ghosh, Sane, Ganapathi, & Nath, 2010). The generation of energy by the respiratory system may produce free radicals and oxygen reactive species at the end of the electron transporting chain (Masibo & He, 2008). Mango fruit may need active antioxidants defense mechanisms to avoid oxidative stress, thus activating the synthesis of phenolic compounds, especially synthesis of gallic acid and chlorogenic acid by the phenylpropanoids pathway. However, further studies related to enzymes responsible from phenolic biosynthesis during ripening of 'Ataulfo' mango and also expression of the genes encoding these enzymes would be useful to understand and explain the metabolic changes of these compounds during ripening.

4. Conclusions

Chlorogenic, gallic, vanillic and protocatechuic acids were the major phenolic compounds in 'Ataulfo' mango pulp, and tended to increase with fruit ripening, chlorogenic acid being the most abundant. On the other hand, the antioxidant contribution of the four phenolic acids increased during ripening. Gallic acid exhibited the highest antioxidant contribution, followed by chlorogenic acid, probably due to their particular chemical conformation, hydroxyl groups and high content in the fruit. This could indicate that these

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phenolic compounds may have an important role in the antioxidant metabolism of 'Ataulfo' mango ripening, and probably be related to important health benefits to consumers. Results indicate that the consumption of ripened mango is better due to its higher antioxidant status and content of major phenolic acids, which may contribute to human health. As far as we know, this is the first report on 'Ataulfo' mango that evaluates the effect of ripening on the changes of individual phenolic compounds. This information can be useful in determining the possible role of the identified compounds that can participate in the prevention of different health disorders. Further studies are needed to evaluate the bioabsorption, biodisponibility and interactions between these compounds present in mango pulp, after consumption.

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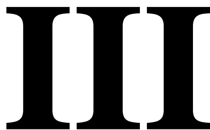

ANTIOXIDANT INTERACTIONS BETWEEN MAJOR PHENOLIC COMPOUNDS FOUND IN 'ATAULFO' MANGO PULP: CHLOROGENIC, GALLIC, PROTOCATECHUIC AND VANILLIC ACIDS

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Communication

Antioxidant Interactions between Major Phenolic Compounds Found in 'Ataulfo' Mango Pulp: Chlorogenic, Gallic, Protocatechuic and Vanillic Acids

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Abstract: Phenolic compounds are known to have antioxidant capacity; however, there is little information about molecular interactions between particular phenolics found in fruits at different developmental stages. Therefore, the total antioxidant capacity of the phenolic compounds of a fruit may not correspond to the sum of individual antioxidant capacity given by antioxidants from that tissue. In this study, individual antioxidant capacity and the interactions of four major phenolic compounds (chlorogenic, gallic, protocatechuic and vanillic acid) found in 'Ataulfo' mango pulp were tested using the DPPH assay. Significant synergism was found in the majority of the all combinations, as well as the combination of the four phenolics. However, antagonism was also observed between some molecules. This work demonstrated particular interactions that may occur in a complex environment within the complex framework of a natural food. The present results may also assist in the future design of functional foods or ingredients based on their antioxidant activity and their synergistic or antagonist interactions.

Keywords: mango; antioxidants; phenolic acids; interactions

1. Introduction

Phenolic acids are antioxidant molecules that are in the limelight of clinical and epidemiological research because their demonstrated value as the antioxidant components of fruits and vegetables [1]. These foods also contain a wide variety of antioxidant bioactive compounds (carotenoids, vitamins, among others) that provide health benefits to consumers [2–5]. Mango (*Mangifera indica* L.) fruit is an excellent source of dietary antioxidants, such as ascorbic acid, carotenoids, and especially phenolic compounds [6]. The health benefits have been demonstrated *in vivo* because of their remarkable antioxidant capacity (AOXC) [7,8].

Recently, it was reported that mango 'Ataulfo' had the highest phenolic content and AOXC among several mango cultivars [9]. According to Palafox-Carlos *et al.* [10], the major phenolic compounds found in 'Ataulfo' mango pulp are chlorogenic, gallic, protocatechuic and vanillic acid. Consumption of these phenolic acids has been found to have an inverse relationship with the incidence of various diseases and chlorogenic and gallic acids may be closely related to those benefits for consumers [9].

The relationship between phenolic bioactive compounds, their AOXC [11] and the health benefits are well established. However, information about phenolic acids and their interactions on the AOXC is scarce. A previous study reported individual phenolic changes during ripening and affected to different extent the AOXC in durian (*Durio* sp.) fruit [5].

Each phenolic compound has a different AOXC depending on its structure, number of aromatic and hydroxyl groups and their distribution in the structure [12,13]. In foodstuffs the composition of bioactive phenolics is complex and it is assumed that all account to the overall AOXC [14]. However, interactions between phenolics could be happening and they could be additive, synergistic or even antagonistic. To gain further insights about interactions in mango pulp, we sought to evaluate the individual and combined antioxidant activities of the four major phenolics in this fruit, in order to provide the bases towards rationally designed nutraceuticals.

2. Results and Discussion

The individual AOXC of the major phenolic compounds in mango 'Ataulfo' was determined and it is shown in Figure 1. The AOXC for each phenolic compound at determined at 0.2 mM. The assay was done using 2,2-diphenyl-1-picrylhydrazy (DPPH) radical and it is reported as percentage of radical scavenge capacity (RSA) (see Experimental section below).

Gallic acid (A) had the highest antioxidant capacity with 61% RSA, followed by protocatechuic acid (B), 35% RSA; chlorogenic acid (C), 28% RSA and vanillic acid had the lowest value of 11% RSA. Our results are similar to those reported by Rice-Evans *et al.* [15], where gallic acid exhibited the highest AOXC, and vanillic acid the lowest among several phenolic compounds. In particular, phenolic acids are considered to be efficient hydrogen donors due to their characteristic carboxylic group, which is easily ionized [16]. Evaluating the individual antioxidant potential of phenolic compounds is a topic that has taken attention in our laboratory in order to understand real potential and

biological action of phenolic antioxidants. In Figure 2 are shown the chemical structures of the four phenolic acids studied. When we compared the structures of phenols of one aromatic ring and the AOXC, the number of hydroxyl groups correlated positively with antioxidant capacity against DPPH. Thus gallic acid presented the highest antioxidant capacity and had four hydroxyls, followed by procatechiuic acid with two and vanillic acid with one hydroxyl group.

Figure 1. Individual antioxidant capacity of phenolic acids at 0.2 mM. Gallic acid (**A**), chlorogenic acid (**B**), protocatechuic acid (**C**) and vanillic acid (**D**).

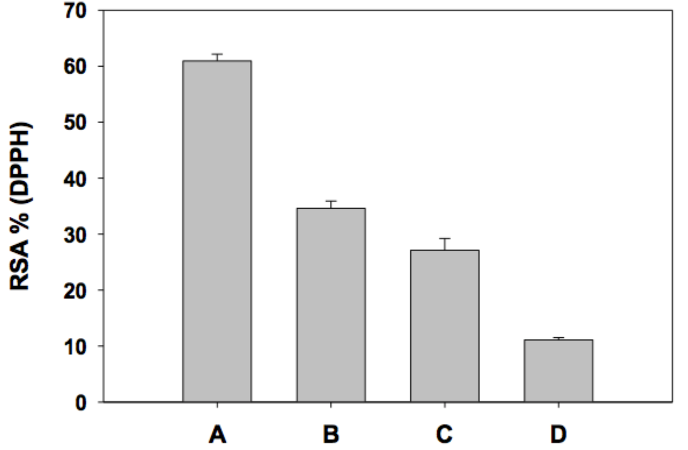
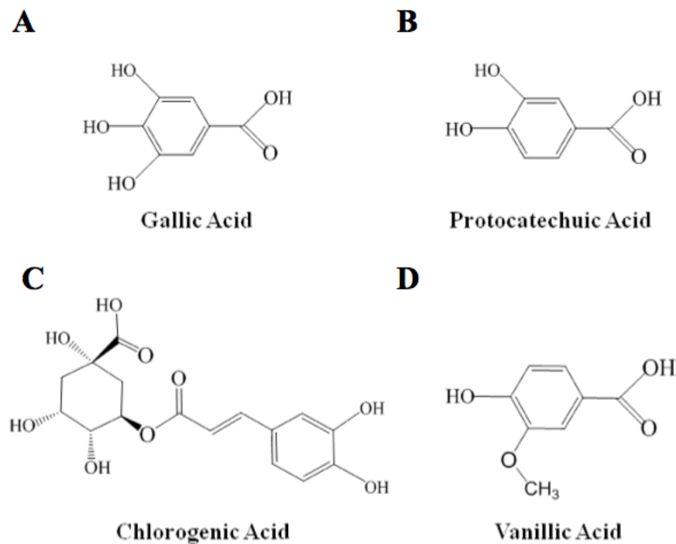


Figure 2. Chemical structure of the compounds studied in this work.



In the case of chlorogenic acid, it has a more complex structure with two hydroxyl groups bound to the aromatic group and four others are bound to a saturated six-member ring. Probably, some steric restrictions among their hydroxyl groups are responsible for being ready for donation to the free radical [17]. Another reason is that the hydroxyl groups in gallic and procatechuic acids are in *meta* position with respect to the carboxylic group, whereas in vanillic acid the OH is in *para* position with respect to the COOH group.

Many studies about the antioxidant potential of phenolic compounds in fruits or foods have concluded that it is impossible to predict the antioxidant power of a given product by studying just one

type of phenolic compound or other kind of antioxidants contained in the product, such as vitamin C or E. In some cases the possible existence of synergistic or antagonistic effects between the various antioxidants present in plant foods and derived products has been discussed [18].

Therefore, we measured the antioxidant activity of individual phenolics and mixes of phenolic acids. Table 1 shows the AOXC of several phenolic acid mixes, representing all possible pair combinations between the four molecules. According to the first combination, between gallic and protocatechuic acid (AB, Table 1-I), where the final concentration of each phenolic was 0.1 mM, the antioxidant arithmetical additive value (47.77 ± 3.1% RSA) was significantly lower ($p \leq 0.05$) than the experimental AOXC value (67.58 ± 2.8% RSA) determined by DPPH assay. This indicates that there is a synergistic interaction between gallic and protocatechuic acid, where the two antioxidants had a higher AOXC compared to a simple additive contribution of each compound (Figure 1). In a similar way, all other paired combinations showed the same pattern, except AD, which reflected an antagonistic interaction since the arithmetic additive value (36.02 ± 1.3% RSA) was significantly higher ($p \leq 0.05$) value than the experimental AOXC value (33.03 ± 1.5% RSA). Combinations BD and CD had no significant difference when compared to the arithmetical addition of individual values, and again vanillic acid (D) was involved in such interactions. The chemical structures suggest that the ether group only found in vanillic acid (D) may be related to the lack of hydrogen transfer required for AOXC.

Moreover, triple combinations of phenolic acids are shown in Table 1-II and their AOXC. Three out of four combinations had a synergistic interaction (ACD, ABC, ABD), while only the combination between protocatechuic-chlorogenic-vanillic acid (BCD) had a small antagonistic interaction. Finally, the AOXC of the combination of four phenolic acids is shown in Table 1-III, since these four phenols are found in 'Ataulfo' mango pulp. The combination ABCD had an experimental AOXC value of 39.76 ± 2.3% RSA, which is significantly higher ($p \leq 0.05$) compared to the theoretical additive value calculated as 33.44 ± 1.7% RSA. These results suggest that these four phenolic acids are interacting in a synergic way in mango pulp and probably in other food systems, but this should be tested.

Table 1. Antioxidant capacity of mixtures containing two, three and four phenolic acids. (**A**) gallic acid, (**B**) protocatechuic acid, (**C**) chlorogenic acid, (**D**) vanillic acid. Different letter at each line indicates significant differences ($p \leq 0.05$).

I.	**Individual (0.1 mM)**	**% RSA Real**	**% RSA Theoretical (Sum)**	**Type of Interaction**
	A	30.47 ± 1.8		
	B	17.30 ± 1.3		
	C	13.56 ± 0.8		
	D	5.55 ± 0.2		
	Combination			
	AB	67.58*a*	47.77*b*	Synergic
	AC	44.96*a*	44.03*b*	Synergic
	BC	34.83*a*	30.86*b*	Synergic
	AD	33.03*a*	36.02*b*	Antagonist
	BD	23.66*a*	22.85*a*	
	CD	20.46*a*	19.11*a*	

Table 1. *Cont.*

II.	Individual (0.066 mM)	% RSA Real	% RSA Theoretical (Sum)	Type of Interaction
	A	20.11 ± 1.3		
	B	11.42 ± 0.7		
	C	8.95 ± 0.4		
	D	3.66 ± 0.5		
	Combination			
	ACD	58.10*a*	32.72*b*	Synergic
	ABC	43.03*a*	40.48*b*	Synergic
	ABD	42.52*a*	35.19*b*	Synergic
	BCD	19.70*a*	24.03*b*	Antagonist
III.	**Individual (0.05 mM)**	**% RSA Real**	**% RSA Theoretical (Sum)**	**Type of Interaction**
	A	15.23 ± 1.1		
	B	8.65 ± 0.8		
	C	6.78 ± 0.6		
	D	2.77 ± 0.2		
	Combination			
	ABCD	39.76*a*	33.44*b*	Synergic

Only a few studies have focused on the assessment of phenolic interactions in terms of antioxidant activity. Heo *et al*. [13] did not find any synergistic effect between the assayed flavonoids by using the ABTS method and expressing results as a vitamin C equivalent. However, Pinelo *et al*. [19] found an antagonistic effect when phenols interacted at three different temperatures using the DPPH method and several studies showed a synergistic antioxidant effect of flavonoids on free-radical-initiated peroxidation of linoleic acid [20].

An antioxidant effect was observed by Pignatelli *et al*. [21] with the flavonoids quercetin and catechin, indicating that these components of red wine act synergistically to inhibit platelet adhesion to collagen and collagen-induced platelet aggregation by virtue of their antioxidant effect.

A common theme in the scientific literature is that interactions between antioxidant molecules do occur, but a mechanism that allows a prediction of synergistic and antagonistic interactions is not apparent. The kind of interaction depends greatly of the specific antioxidants interacting in the system and the condition behind the evaluation [22]. In our case, more than the 80% of our phenolic combinations showed synergistic interactions. Our results suggest that these phenolic acids are capable not only to donate hydrogen atoms to the radical, but they are also able to donate electrons to regenerate other pro-oxidant phenols. This regeneration mechanism maximizes the AOXC of the system to reduce free radicals. According to Leopoldini *et al*. [16], phenolic compounds are capable to transfer electrons to other phenolics or antioxidants, promoting their chemical regeneration.

In summary, synergistic interactions occurred between the major phenolic acids found in mango ‘Ataulfo’. Based on these results, the importance of choosing the best combination of antioxidants may be advantage when designing new dietary supplements or nutraceuticals.

3. Experimental

Pure commercial standards of gallic, chlorogenic, protocatechuic and vanillic acid were used for all experiments (Sigma-Aldrich, Toluca, Mexico). AOXC was determined by DPPH, and reported as percentages of radical scavenger capacity (RSA). The DPPH assay was conducted according to the method reported by Brand-Williams *et al.* [23] with some modifications. The DPPH solution was adjusted at an absorbance of 1.0 ± 0.02 at 515 nm. Samples of 10 µL were placed in a microplate and 140 µL of DPPH radical were added. After an incubation of 30 min the samples were read at 515 nm using an Omega spectrophotometer (BMG Labtech Inc., Ortenberg, Germany).

To determine the synergistic or antagonistic interactions between the mango phenolic acids, gallic (A), protocatechuic (B), chlorogenic (C) and vanillic (D) acids were prepared as 0.2 mM concentration stock solutions in 80% methanol. All possible combinations were established. The combinations of phenolic acids were grouped in three sets: combination of two phenolic acids (CB2), combination of three (CB3) and finally the combination of the four phenolic acids (CB4). Each combination was mixed on an equal one mL volume basis maintaining same proportion between the phenolic acids in the mix. The AOXC of each combination was determined using DPPH method as described above. The AOXC of individual phenolic acid at final concentration at each combination were determined to calculate the theoretical value of the mix. This value was established as the sum AOXC values of the individual phenols in each mix. The real AOXC exhibited in each mix was established as the real value. Thus, the theoretical and real values were compared in order to determine if significant synergistic or antagonistic interactions occurred. Results were expressed as means and indicating literals indicate significant differences. Data were statistically analyzed by one-way ANOVA procedure, and the Tukey-Kramer multiple comparison test was used. Standard deviation and variance coefficient between data groups were used to determine significant differences between them at $p \leq 0.05$ using the statistical software Statgraphics Plus for Windows® v. 5.0. Four replicates were used for each experiment

4. Conclusions

Gallic and protocatechuic acid exhibited the highest antioxidant capacity, probably due to their particular chemical conformation and hydroxyl groups content. According to our observations, the phenolic acids present in a mixture can interact, and their interactions can affect the total antioxidant capacity of a solution. It can also be concluded that there are synergistic interactions between the major phenolic acids present in mango 'Ataulfo', excluding vanillic acid, which appears to have a negative effect. In the light of the results presented here, the importance of choosing the best combination of antioxidants should be taken in consideration when designing functional foods. More studies with combinations are required in a more mechanistic way, including infrared spectrometry and magnetic nuclear resonance, in order to better understand the mechanisms that are taking place inside an antioxidant system. Also, further studies are needed to evaluate the bio absorption, bioavailability and interactions between these compounds present in mango pulp, after consumption.

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IV

EXPRESSION AND ENZYMATIC ACTIVITY OF PHENYLALANINE AMONIO-LIASE AND *P*-COUMARATE 3-HYDROXYLASE IN MANGO (*Mangifera indica* var. Ataulfo) DURING RIPENING

En preparación para: Journal of Plant Physiology

Introduction

Fruit ripening is a developmentally regulated process resulting from the coordination of numerous biochemical and physiological changes within the fruit tissue that culminates in changes in firmness, color, taste, aroma, and texture of fruit flesh (Singh et al. , 2010, Vishwas A. Bapat et al. , 2010). Phenolic compounds produced by the phenylpropanoid pathway contribute to fruit pigmentation, as well as to the response derived from disease, insects attack and stress resistance found in many fleshy fruits during ripening, among other metabolic roles (Boudet, 2007). The phenylpropanoids metabolism is a representative and essential biosynthetic pathway in all plant cells, but the regulation of all genes involved is particular of the type of plant tissue (Singh, Rastogi, 2010). The relation between fruit ripening process and the biosynthesis of phenolic compounds is a complex topic to approach, and exits several interrogations about it nowadays (Rinaldo et al. , 2010). However, some studies reported evidences that ripening process affect directly the phenylpropanoids pathway (Palafox-Carlos et al. , 2012a, Singh, Rastogi, 2010).

There are several key enzymes which are involved in the phenylpropanoid pathway namely, phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), hydroxycinnamate 3-hydroxylase (C3H), 4-coumarate CoA ligase (4CL), among others such *O*methyltransferase (OMT) (Vogt, 2010). The role of PAL is to drive the carbon flow from the aromatic amino acid *L*-phenylalanine (*L*-Phe) for the production of 4-coumaroyl-CoA (Rinaldo, Mbéguié-A-Mbéguié, 2010, Rosler et al. , 1997). In the case of C3H, it role is implicated in the hydroxylation (attaching of OH groups) in the 3´ carbon of the aromatic ring in diverse phenol intermediates, but especially in the hydroxylation of *p*-coumaric acid to form caffeic acid (Abdulrazzak et

al. , 2006, Nair et al. , 2002). In this sense, the role of gene *PAL* and *C3H* are necessary for the biosynthesis of almost all phenolic compounds in nature.

Mango (*Mangifera indica L.*) fruit can be considered not only an economically important fruit worldwide, but also a good source of dietary antioxidants, such as ascorbic acid, carotenoids, and especially phenolic compounds (Ma et al. , 2011), which have demonstrated different health-promoting properties, mainly due to their remarkable antioxidant capacity (Gonzalez-Aguilar et al. , 2010). It was reported that ´Ataulfo´ mango had the highest phenolic content and antioxidant capacity among several mango varieties (Manthey and Perkins-Veazie, 2009). Furthermore, our research group recently reported that ´Ataulfo´ mango showed a characteristic high content of phenolic acids, and the antioxidant capacity of this fruit correlated to its total phenolic content and composition (Palafox-Carlos, Yahia, 2012a, Palafox-Carlos et al. , 2012b). Consequently, it would be convenient to evaluate important genes involve in the phenolic acid biosynthesis such *PAL* and *C3H*, in order to understand and /or estimate their participation during ripening of mango fruit.

Materials and methods

Plant material

Fresh mango fruit (average weight of 200-300g) (*Mangifera indica* L. cv. Ataulfo) were harvested from a field in Tepic, Nayarit, Mexico, and transported immediately to the laboratory for evaluation. Fruit were selected according to their size, color and appearance discarding fruit with defects and physiological disorders. Afterwards, fruit were sanitized with chlorinated water (200 ppm sodium hypochlorite) for 3 min and left to dry at room temperature (23-26°C) for about 1 h. Fruit were selected according to peel surface color, and divided in 4 groups, including 16 fruits each. A total of four

ripening stages (RS) were established as: RS1, representing mango with a yellow surface area of 0-10%; RS2, 20-30%; RS3, 70-80% and RS4, 100% yellow color. The pulp was removed, cut into small pieces as quickly as possible, immediately frozen in liquid nitrogen and storage at -80°C, afterwards.

RNA isolation and complementary DNA synthesis

Total RNA was isolated from the mango mesocarp tissue according to Lopez-Gomez and Gomez-Lim (Lopez-Gomez and Gomez-Lim, 1992), with some modifications. The RNA quantity was estimated at 260 nm using a Nano-Drop ND-1000 UV-Vis spectrophotometer (Nano Drop Technologies Inc., Wilmington, DE, USA). The RNA integrity was detected by agarose gel electrophoresis under denaturing conditions. The cDNA synthesis was performed by reverse transcription from 5 µg of total RNA, using the SuperScript II kit (Invitrogen, Carlsbad, CA, USA).

Expression by Real-Time quantitative PCR

The quantitative PCR was carried out using iQ™ SYBR® Green Supermix (BIO-RAD). All samples were PCR-amplified in triplicates in reactions, which included 25 ng of cDNA as template; 12.5 µL of SYBR ® Green qRT-PCR Master Mix; 1 µL of 5 µM sense primer; 1µL of 5 µM antisense primer and water to 25 µL final volume. Specific primers used in qPCR for *PAL* were sense 5'-GGCTGCAGCAATTATGGAAC -3' and antisense 5´-ACTTCAATCAGTGGGCCAAG -3'; for *C3H* were sense 5'-GGGTTGAAACTTGGAGCTTC -3' and antisense 5'-GACGAAATGATGCTTGACACC-3'; for *GAPDH* were sense 5'-GTGGCTGTTAACGATCCCTT-3' and antisense 5'-GTGACTGGCTTCTCATCGAA-3'. The PCR products were amplified in a Step-One™ Real-time PCR System (Applied

Biosystems). The amplification conditions were 40 cycles including sequentially 95°C for 5 min, 95°C for 15 s, 60°C for 1 min and 72°C for 5 min. PCR product specificity was confirmed by constructing a melt curve after amplification raising temperature from 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Non-template controls were included during each gene amplification. The method $2^{-\Delta\Delta C}$T was used to evaluate changes in the relative mRNA amount of target genes (Livak and Schmittgen, 2001). Data was analyzed based on the CT value of each sample during PCR amplification, where –ΔΔCT= –((CTtarget-CTGAPDH)-(CT*Avg*target-CT*Avg*GAPDH)), and *Avg* corresponded to the averaged CTs from runs of day 1. The results were expressed as relative mRNA steady-state levels of the target gene and normalized to the *GAPDH* expression levels. Statistical analysis was performed by one-way ANOVA, with a 0.05 significance level, using the NCSS (2007) software.

Enzymatic activity assays

The crude extract for both, PAL and C3H activity analysis, was prepared using 0.2 g of mango mesocarp tissue, mixed with 0.2 g PVPP (PolyVynilPolyPyrrolidone) and homogenized in 25 ml of cold 5 mM borate buffer (pH 8.5) at low speed during 1 min. The suspension was centrifuged at 15,000 X *g* (Beckman Coulter, Allegra 62R, USA) for 15 min at 4°C. The supernatant was collected and incubated during 5 min at 40°C. The enzymatic reaction was performed with 1.9 ml extract and 100 µl of phenylalanine 100 mM, as a substrate. The mix was incubated for 1h at 40°C. After having it incubated, the mix was collected and absorbance was read using an Omega spectrophotometer (BMG Labtech Inc., Germany) with a microplate reader device, at a 280 nm wavelength. The values were calculated as µmoles of cinnamic acid/h per g of fresh weight (FW).

The assay for *p*-coumaroyl 3-hydroxylase (C3H) activity was performed according to Schoch et. al (2001), with some modifications. An incubation mixture was prepared in a final volume of 500 µl, containing 0.5 mM *p*-coumaric acid, 50 mM potassium phosphate (pH 7.0), 250 µl of the crude extract, and it was incubated for 1 h at 28 °C in the dark. The reaction was terminated by addition of 60 µl of acetic acid and centrifuged at 18,000 X *g* for 15 min, at 4°C. The supernatant was collected and absorbance was read at 325nm using an Omega spectrophotometer (BMG Labtech Inc., Germany). The activity was reported as µmoles of caffeic acid/h per g FW.

Results

The relative expression of *PAL* and *C3H* was evaluated during ripening of mango ´Ataulfo´ fruit, as shown in figure 1; both *PAL* and *C3H* were differentially expressed through fruit ripening. The highest relative expression was reached by *PAL* at RS1 and RS4 (about 1-fold), with no significant differences; the lowest expression was reached at RS2 (0.1-fold) with a significant increase at RS3. Regarding to *CH3*, the relative expression increased from RS1 to RS3 and then significantly decreased in RS4. The lowest relative expression of *CH3* was observed at RS2 (0.1-fold), and the highest at RS3 (1.4-fold).

Conversely, the PAL and CH3 enzymatic activity was also evaluated during mango fruits ripening (figure 2A and 2B, respectively). No significant differences in RS1 and RS2 (about 9 µmoles cinnamic acid/h/ FW) were revealed by the PAL activity, but it increased at RS3 though, with no significant difference with RS4 (about 12 µmoles cinnamic acid/h/ FW). Concerning to C3H activity, the pattern was similar to the *C3H* expression, since enzyme activity increased from RS1 to RS3, with a significant decrease at the RS2. Besides, the highest enzymatic activity was observed at

RS3 (11.85 µmoles caffeic acid/h/ FW). It is important to point out, that mango as a climacteric fruit, showed the climacteric peak (highest respiration rate) at RS3 (data not shown). Also, these RS were according to those reported previously by our research group (Palafox-Carlos, Yahia, 2012a).

Discussion

Phenolic compounds produced by the phenylpropanoid pathway contribute to fruit pigmentation, as well as to the disease resistance response found in many fleshy fruits ripening (Boudet, 2007). The relation between the fruit ripening process and the biosynthesis of phenolic compounds is a complex topic to study, and several interrogations about it these days come up (Rinaldo, Mbéguié-A-Mbéguié, 2010). However, the phenylpropanoids pathway related with the biosynthesis of phenolic compounds have been recently reported to be directly affect by the ripening process (Palafox-Carlos, Yahia, 2012a, Singh, Rastogi, 2010).

Despite the magnificent diversity in genes regulation, certain genes such as *PAL, C4H*, *C3H, 4CL*, among others, are considered key genes involved in the phenylpropanoid pathway (Vogt, 2010). The present study is focused on the *PAL* and *C3H* genes evaluation, and the enzymes activity on mango ´Ataulfo´ ripening.

The *PAL* expression was higher at the beginning and at the end of the ripening, reflecting this gene activation o in two different metabolic moments of the mango fruit ripening. Most of the necessary secondary metabolites involved in ripening need to be synthesized in RS1, including both the phenolic compounds and lignin, specially (Giovannoni, 2004). This observation is in accordance with Shan et al. (2008), who suggested that the high *PAL* expression during fruit development early stages, is related with either the considerable increase in vascular tissues formation or structural modification.

Conversely, the expression of *PAL* becomes diminished after RS1, starting to increase from RS3 until it reached the maximum expression value at RS4. A high respiration rate was revealed by the fruit from RS3 (climacteric peak) to RS4 (Palafox-Carlos, Yahia, 2012a), which would produce reactive oxygen species (ROS) (Palafox-Carlos, Yahia, 2012b). The plants mechanism to overcome this oxidative stress (or senesce) is triggered by increasing the biosynthesis of phenolic compounds (Gonzalez-Aguilar, Villa-Rodriguez, 2010). This may be the reason why the *PAL* expression is up-regulated at the end of mango ripening.

Regarding PAL enzymatic activity, in contrast to *PAL* expression, the enzyme is active during all ripening; with a significant increase in the last two mango fruits RS. The expression does not correspond to the enzyme activity, suggesting that genes and enzymes regulation is taking place in different levels, thus, our results are in accordance with Promyou et al. (2007), who reported a lack of a correlation between the gene expression and the PAL for ´Sucrier´ banana. An increase in PAL activity, in the case of Cherimoya fruit (*Annona cherimola*), was also exhibited, without a significant increase in other phenylpropanoids compounds biosynthesis, even though such enzyme is a key part of the phenylpropanoid pathway (Assis et al. , 2001). Consequently, due the biological complexity of metabolism *per se*, it is important to point out that the principle of one gene leads to one protein leads to one metabolite is a simplistic and often incorrect notion, as experimentally demonstrated (Mutch et al. , 2005).

Until now, it has been difficult to fully understand the *PAL* role either, or its enzymes isoforms, nor their regulation. The *PAL* gene has been cloned, as well as characterized from many plant tissues (Boudet, 2007); it seems to exist universally in higher plants as a family of genes; therefore, the *PAL* isoforms presence is a common observation. The significance of this diversity is unclear; nonetheless, t it is suggested

by evidence, for a degree of metabolic channeling within phenylpropanoid metabolism, that partitioning into phenylpropanoid metabolism particular branches may involve labile multienzyme complexes, including PAL specific isoforms (Sreelakshmi and Sharma, 2008). As an instance, fruit color and flavor development in raspberry (*Rubus idaeus*) ripening relied on PAL encoded by a family of 2 genes (*ripal1* and *ripal2*). The *ripal1* gene was associated with early fruit ripening events, whereas expression of *ripal2* was more easily correlated with flower and fruit development later stages (Kumar and Ellis, 2001).

A *PAL* absolute expression in all RS was evaluated in the study hereby. Concerning climacteric fruits, such as´Ataulfo´ mango, the ethylene production during ripening is quite remarkable (Palafox-Carlos, Yahia, 2012a), and it may be a PAL activity regulator. The presence of ethylene in accordance to Cai et al. (2006), is not only required for PAL enzyme synthesis, , but also to maintain its continuous high activity, according to the results revealed by the present study. Although, in this study on mango fruits, differences in chemistry were revealed in all of their ripening stages; moreover, the determination of the exact role played by each isoform in supporting accumulation of specific phenylpropanoid products in fruits, would require a detailed metabolite profiling.

The highest expression level in the case of *C3H* was reached at RS3, having its pattern corresponding with the C3H enzymatic activity, showing the highest activity in the climacteric peak. The positive correlation between phenylpropanoids genes expression and corresponding enzymes is also an expected result, as reported in several fruits, especially in ´berrys´ (Jaakola et al. , 2002), strawberries (Almeida et al. , 2007) and banana (Chen et al. , 2008), among others (Pandit et al. , 2010, Sanchez-Ballesta et al. , 2000, Yingsanga et al. , 2008); this indicates that both genes and proteins are

regulated at the same level. According to recent reports by Palafox-Carlos et al. (2012a, 2012b), the highest total phenol value in ´Ataulfo´ mango pulp was shown at RS3; the major phenolic compounds found were phenolic acids. Chlorogenic and gallic acid were the most abundant; the highest content was found in the climacteric peak (Palafox-Carlos, Yahia, 2012b). The *C3H* has been reported as a gene responsible for phenolic acids specific biosynthesis (Abdulrazzak, Pollet, 2006, Mahesh et al. , 2007). Hence, the results present in this study are in agreement to those recently reported in ´Ataulfo´ mango. This would explain why the highest *CH3* expression is in accordance to the highest phenolic acid content in mango fruit at the climacteric peak.

The C3H was originally named after its suspected function of C3-hydroxylation of *p*-coumaric acid and production of caffeic acid, (Franke et al. , 2002, Schoch, Goepfert, 2001); however, it is now known that this enzyme participates in other several steps involved in   phenolic acids and derivatives biosynthesis (Assis, Maldonado, 2001, Mahesh, Million-Rousseau, 2007). Most of the reports in literature have been done on other plant tissues, such as *Arabidopsis* (Schoch, Goepfert, 2001), *Coffea canephora (Mahesh, Million-Rousseau, 2007)*, *Gingo biloba* (Liu et al. , 2008) or on C3H over expressing yeast (Nair, Xia, 2002), just to name a few. Accordingly, as far as we know, this is the first report examining C3H in fruits ripening, in tropical fruits.

The PAL enzyme has a major role in plants and the phenolypropanoids biosynthesis, in comparison to C3H; not only because PAL initiates the phenylpropanoids pathway, but also because this pathway is responsible for production of other important secondary metabolites, necessary in plant-cells life, such as coumaroin, lignin, terpenoids, etc (Cai, Xu, 2006, Vogt, 2010). As a consequence, this may be the reason the PAL enzyme   always revealed activity in all mango ripening.

Consequently, the relationship of the expression and enzyme activity data found of mango cv. ´Ataulfo´ physiological parameters analyzed in this study during the maturation reveals evidence of the role performed by *PAL* and *C3H* genes associated with this process. This study emphasizes the complex nature of phenolic compounds regulation in tropical fruits, at least at the biosynthetic gene level. We hope this work gives valuable information to future researchers in understanding emerging concepts in the regulation of phenolic compounds biosynthesis during ripening of tropical fruits.

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Figure legends

Figure 1. Relative expression of *PAL* and *C3H* in mesocarp of ´Ataulfo´ mango fruit during ripening.

Figure 2. Enzymatic activity of PAL and C3H in mesocarp of ´Ataulfo´ mango fruit during ripening.

Figure 1

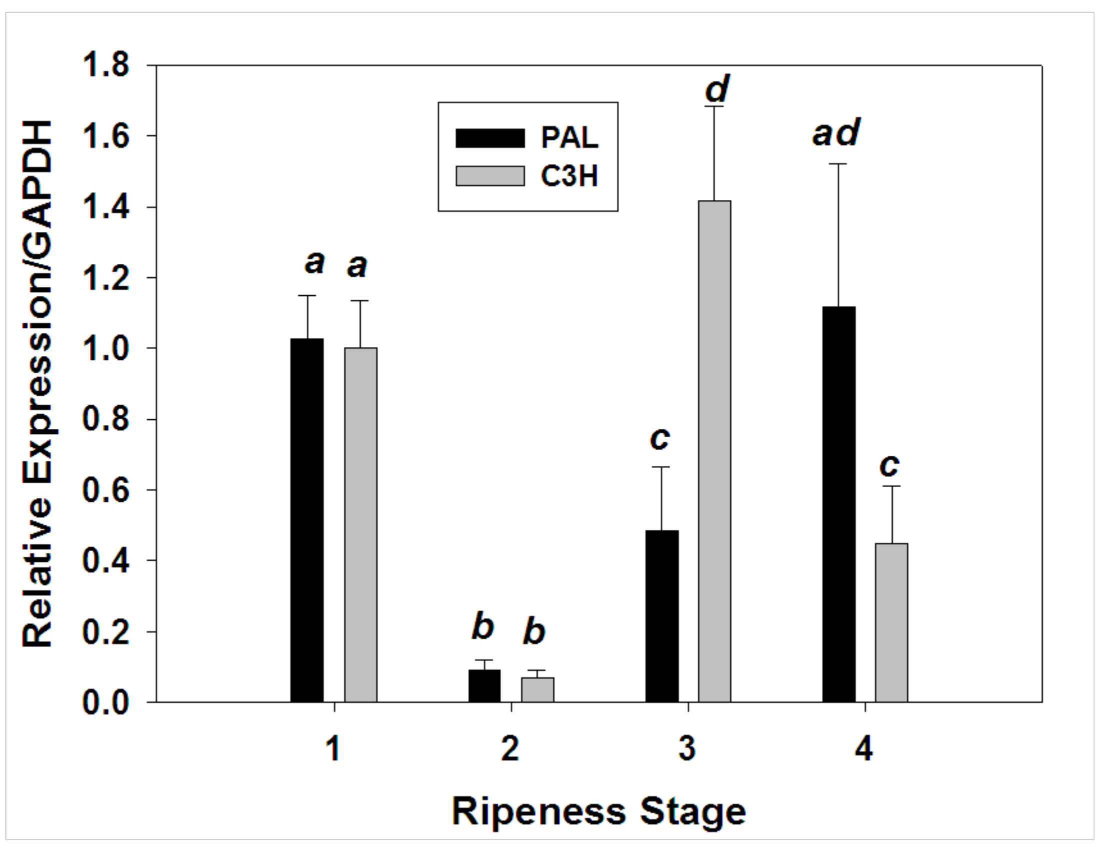
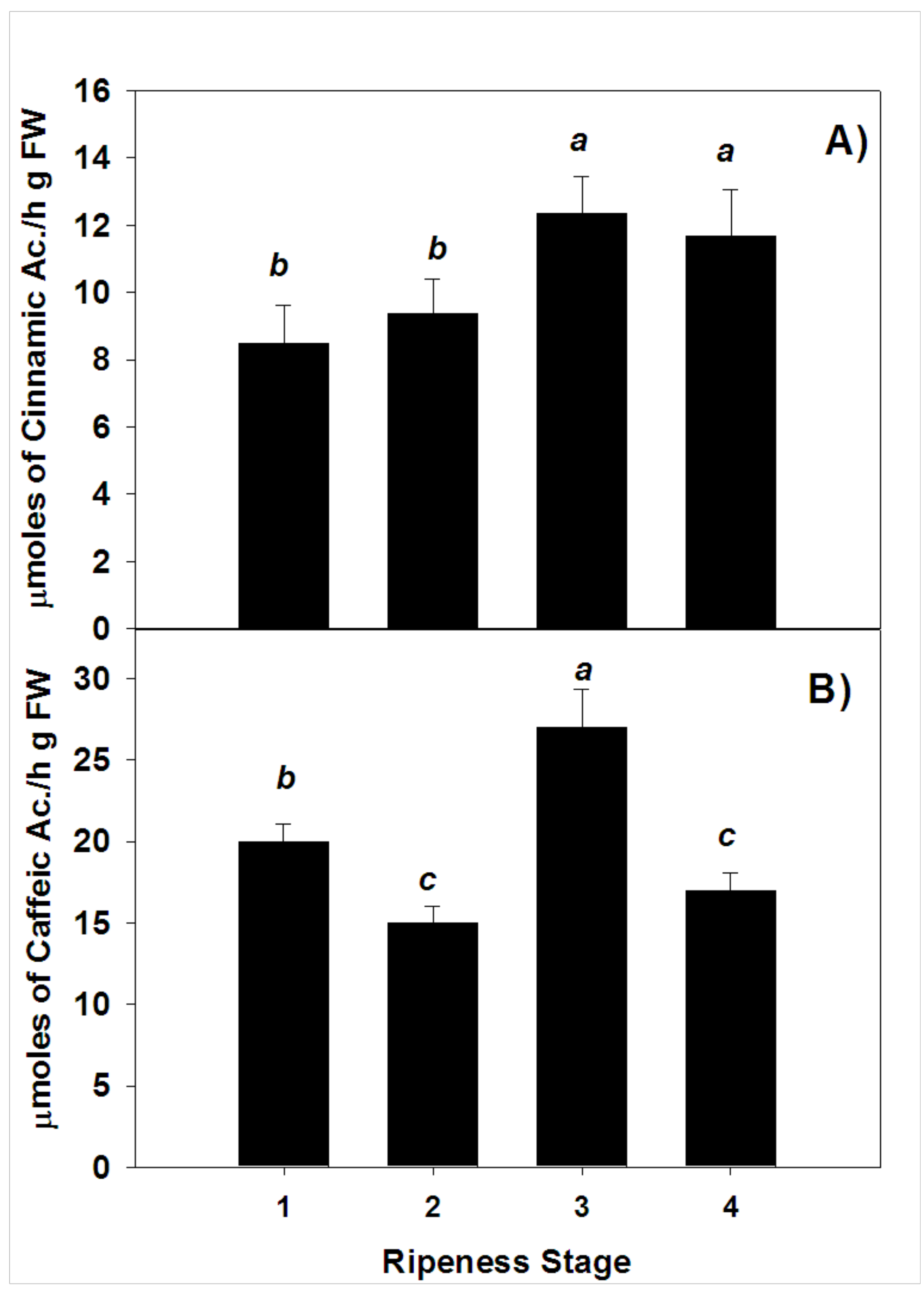


Figure 2



CONCLUSIONES GENERALES

A partir de los resultados derivados de este estudio se tienen las siguientes conclusiones:

1. Existe una correlación positiva entre la respiración del fruto, fenoles totales y la capacidad antioxidante durante la maduración de mango ´Ataulfo´, por lo que los estados de madurez 3 y 4 son los que mostraron los mayores valores de estas 2 variables.
2. Los ácidos fenólicos son los compuestos fenólicos predominantes en la pulpa de mango ´Ataulfo´, donde ácido clorogénico es el más abundante, seguido de gálico.
3. El contenido de fenoles totales y la contribución de los principales compuestos fenólicos aumenta con la maduración en mango ´Ataulfo´, lo cual nos indica que los estados de madurez más avanzados aportan una mayor cantidad de compuestos bioactivos y como consecuencia mayores beneficios para la salud.
4. Se comprobó que los ácidos fenólicos presentaron diferente capacidad antioxidante, siendo ácido gálico el de mayor capacidad, seguido de ácido protocateico, lo cual es atribuido a su estructura química y distribución de los grupos OH, los cuales son determinantes en la capacidad para donar sus electrones para estabilizar los radicales libres.
5. El potencial antioxidante característico del mango ´Ataulfo´ medido *in vitro*, al parecer está en función del efecto sinérgico de sus principales cuatro ácidos fenólicos. Sin embargo, otros compuestos hidrofílicos participan en un porcentaje importante en esta variable
6. El gen *PAL* y la enzima phenilalanina amonio liasa presentaron un rol muy activo durante la maduración, en especial la enzima. Sin embargo, de acuerdo a los resultados obtenidos ambas parecen estar

reguladas a diferente nivel. Por su parte, el gen *C3H* presentó una correlación positiva con la enzima *p*-cumarato 3-hidroxilasa, y ambas sugieren estar directamente relacionada con los cambios en el contenido de ácidos fenólicos encontrados en mango ´Ataulfo´.

ANEXOS

PRODUCCIÓN ACADÉMICA

Artículos Publicados con Arbitraje

Hugo Palafox-Carlos, Joana Gil-Chávez, Rogerio R. Sotelo-Mundo, Jacek Namiesnik, Shela Gorinstein and Gustavo A. González-Aguilar. (2012). Antioxidant Interactions between Major Phenolic Compounds Found in 'Ataulfo' Mango Pulp: Chlorogenic, Gallic, Protocatechuic and Vanillic Acids. Molecules, 17, 12657-12664.

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J.F. Ayala-Zavala, G.A. González-Aguilar, J.E. Dávila-Aviña, V. Vega-Vega, **H. Palafox-Carlos**, J.A. Villa-Rodríguez, C. Rosas-Domínguez. (2012). Capitulo 4. Compuestos naturales como antimicrobianos y antioxidantes en alimentos. En Antioxidantes en la industria de los alimentos. Editorial Trillas. Pag. 81-101.

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Estancia en el laboratorio de fitoquímicos y nutrición en la Universidad Autónoma de Querétaro, bajo la dirección del Dr. Elhadi Yahia (SNI nivel III). Se llevó a cabo de Junio a Octubre del 2010 en la ciudad de Querétaro, Qro., México.

Distinciones y Participaciones Especiales

Colaborador en el proyecto de investigación de sicología política desarrollado por el Centro de Gobierno y Estudios Internacionales de la Universidad de Harvard. Bajo la dirección del Dr. Ryan D. Enos. Se llevo a cabo durante el verano del 2012. Cambridge, MA. EUA.

Finalista nacional en el proceso de selección para obtener la beca Fulbright-García Robles, dentro de la convocatoria 2011 para realizar estancias de investigación en EUA. Entrevista final en México DF, llevada a cabo el día 22 de Noviembre del 2011, ante el comité de la fundación Fulbright.

Premio como uno de los tres mejores trabajos orales presentados en el III simposio Brasileiro de Pós-colheita de Frutas, Hortalicas e Flores. Llevado a cabo del 23 al 26 de Octubre del 2011en Nova Friburgo, RJ, Brasil.

Cursos

Asistencia al curso de certificación "Annual General Safety and Hazard Communications, Chemical Hygiene Safety, and Hazardous Chemical Waste Training". Llevado a cabo en Tufts Univeristy, Boston, EUA, el 11 de Junio del 2012.

Asistencia al curso de certificación “Annual Bloodborne Pathogenes Training”. Llevado a cabo en Tufts University, Boston, EUA, el 11 de Junio del 2012.

Asistencia al curso titulado “Compuestos naturales con actividad biológica y su impacto en la salud”. Organizado dentro del VII Congreso de Noroeste y III Nacional en Ciencias Alimentarias y Biotecnología”. Llevado a cabo en la Universidad Autónoma de Sonora, del 8 al 10 de Noviembre del 2010, en Hermosillo, Son, México.

Presentación en Congresos

Palafox-Carlos, H., Yahia, E.M, González-Aguilar, G.A. “Identification and Quantification of Phenolic Compounds from Mango (*Mangifera indica*, cv. Ataulfo) Fruit Determined by HPLC-DAD-MS/MS-ESI and Their Contribution to the Antioxidant Activity during Ripening”. En el III simposio Brasileiro de Pós-colheita de Frutas, Hortalicas e Flores. Llevado a cabo del 23 al 26 de Octubre del 2011en Nova Friburgo, RJ, Brasil. (Presentación oral).

Hugo Palafox Carlos, Yahia Elhadi, Porfirio Gutierrez, Miguel Mata y Gustavo Gonzalez Aguilar. Identificación y cuantificación de compuestos fenólicos durante la maduración de mango ´Ataulfo´. En el XIV Congreso Nacional de la Sociedad Mexicana de Ciencias Horticolas, A.C. Llevado a cabo del 10 al 14 de Abril del 2011. (Presentación de cartel)