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# CHEMICAL CHARACTERIZATION AND QUANTIFICATION OF THE PIGMENT EXTRACTION YIELD OF SEVEN MEXICAN ACCESSIONS OF *Bixa orellana*
# CARACTERIZACIÓN QUÍMICA Y CUANTIFICACIÓN DEL RENDIMIENTO DE EXTRACCIÓN DE PIGMENTO EN SIETE ACCESIONES MEXICANAS DE *Bixa orellana*

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**Abstract**

Achiote (*Bixa orellana*) is a plant used for obtaining a natural dye rich on carotenoids (mainly bixin and norbixin); it is also the plant species with the highest content of tocotrienols in nature. In the present work, the pigment extraction yield of seven Mexican accessions of *Bixa orellana* was quantified. Also color parameters and content of tocotrienols, tocopherols, norbixin, bixin, total phenolic compounds and antioxidant capacity were evaluated in the corresponding annatto extracts. The highest percentage of pigment extraction yield was obtained with KOH (4.84%). Accessions 43 (L*= 4.01 ± 0.79, C*= 7.33 ± 1.07, h= 25.76 ± 6.35) and 50 (L*= 3.17 ± 0.64, C*= 6.81 ± 0.53, h= 26.41 ± 4.41) had the lowest color values, meaning these accessions had a darker and redder color. Four accessions showed the highest content of bixin: accession 48 (3.1%), 45 (2.6%) 43 (2.4%) and 47 (2.2%). Accession 50 had showed the highest content of total phenolic compounds and of tocotrienols (T3), mainly the isoform $\delta$-T3 (5.03 ± 0.64 mg $g^{-1}$ Seed Dry Weight), as well as the highest antioxidant capacity.

*Keywords*: annatto extracts, pigment yield, norbixin, bixin, phenolic compounds.

**Resumen**

El achiote (*Bixa orellana*) es una planta utilizada para obtener un colorante natural rico en carotenoides (principalmente bixina y norbixina); además, es la especie vegetal con el mayor contenido de tocotrienoles. En este trabajo, se determinó el rendimiento de extracción de pigmento de siete accesiones mexicanas de *Bixa orellana*. También se evaluaron los parámetros de color y el contenido de tocotrienoles, tocoferoles, norbixina, bixina, compuestos fenólicos totales y la capacidad antioxidante en extractos de annato. El mayor porcentaje de rendimiento de extracción de pigmento fue obtenido con KOH (4.847905%). Las accesiones 43 (L*= 4.01 ± 0.79, C*= 7.33 ± 1.07, h= 25.76 ± 6.35) y 50 (L*= 3.17 ± 0.64, C*= 6.81 ± 0.53, h= 26.41 ± 4.41) presentaron los valores más bajos de los párametros de color, lo que significa que estas accesiones tuvieron un color más oscuro y más rojo. Cuatro accesiones mostraron el mayor contenido de bixina: accesión 48 (3.1%), 45 (2.6%) 43 (2.4%) and 47 (2.2%). La accesión 50 mostró el mayor contenido de compuestos fenólicos totales y de tocotrienoles (T3), principalmente la isoforma $\delta$-T3 (5.03 ± 0.64 mg $g^{-1}$ Peso Seco), así como también la mayor capacidad antioxidante.

*Palabras clave*: extractos de annato, rendimiento de pigmento, norbixina, bixina, compuestos fenólicos.

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# 1 Introduction

Achiote (*Bixa orellana* L.) or annatto is a plant native to tropical America. It is a perennial tree that can reach nine meters in height with white or pink flowers and fruits with the form of globular ovoid capsules. Each capsule may contain from 30 to 45 ovoid or cone-shaped seeds covered by a thin red-orange layer or viscous aril (Fonnegra and Jiménez, 2007). Natural dyes obtained from the seeds of achiote plant have been used since pre-Hispanic times. Annual world production of achiote seeds is approximately 14, 500 tons (dry weight). Latin America produces 60% of the total world production. The main producers are Peru, Brazil and Mexico. At present, at least 67 accessions have been collected from the Yucatan Peninsula in Mexico in order to preserve and propagate the genetic material of this plant species, although only a few of them have been characterized. An accession is a sample of a distinct germplasm, where germplasm is defined as the set of genes expressed in the distinctive phenotype; therefore, collection of different accessions and its preservation in germplasm banks is very important for conservation of genetic diversity and its potential commercial usage.

Annatto is the name given to the crude pigment extract obtained from achiote, which contains bixin, norbixin and other carotenoids in varying proportions. While bixin is liposoluble, norbixin is hydrosoluble, and the possibility of obtaining both water-soluble and oil-soluble colorants depending on the type of extraction used as well as the solvent and temperature applied has converted achiote into one of the most interesting sources of plant colorants (Toledo de Oliveira *et al*., 2004).

Currently, the use of artificial colorants such as tartrazine (E102), allura red (E129) or sunset yellow FCF (E110) in food products have been severely questioned in developed countries, since there are reports showing that indiscriminate consumption of these colorants is associated to the development of degenerative illnesses such as some types of cancer (Salinas *et al*., 2005). As a consequence, the use of some artificial food colors such as carmoisine (E122) and Ponceau 4R (E124) have been banned in the USA and Europe, while the use of natural colorants such as the dye that is extracted from the surfaces of *Bixa orellana* L. (E 160b, annatto extract) have been recommended as alternatives (Toledo de Oliveira *et al*., 2004; McCann, 2007). The annatto extract has therefore accrued great economic importance worldwide as it is one of the natural dyes most widely used in the food, cosmetic and pharmaceutical industries as it does not alter flavor and it is practically non-toxic (Michelangeli *et al*., 2002; Smith 2006; Lourido and Martínez, 2010).

The natural dye obtained from *Bixa orellana* is rich on the carotenoids bixin (dark red color) and norbixin or orelline (yellow color) which are mainly used to develop attractive colors in dairy products (cheeses, margarine, and butter), meats, ice creams, cosmetics, condiments, ceramic, paint, hair colors, soaps, nail polish, varnish, lacquer, fabric colors, among others (Devia and Saldarriaga, 2003). Bixin represents approximately 80% of the total carotenoids content present in the dye obtained from achiote (mainly in its 9-*cis* configuration with low amounts of *trans*-bixin) (Smith, 2006). Other apocarotenoids present in lower amounts are norbixin, bixin dimethyl ester and byproducts of lycopene degradation (Cardarelli *et al*., 2008). On another hand, it has been documented that achiote (*Bixa orellana* L.) contains important amounts of tocotrienols, tocopherols, terpenes and flavonoids both in the seeds and in the leaves (Rodrigues *et al*., 2007; Chisté *et al*., 2011).

Vitamin E is an essential micronutrient in human and animals diets due to its biological activity and its powerful antioxidant capacity (Cano-Sarmiento *et al*., 2014). Vitamin E is the generic name given to all compounds that exerts the biological functions of $\alpha$-tocopherol (Frank *et al*., 2012). However, vitamin E is a family of lipophilic compounds which includes four tocotrienols (T3) and four tocopherols (Ts) ($\alpha$, $\beta$, $\gamma$, $\delta$) (Aggarwal *et al*., 2010). The structure of these two types of compounds is very similar; but, while tocopherols have a saturated phytol tail, tocotrienols possess an isoprenoid tail with three unsaturated residues (Das *et al*., 2007; Almeida *et al*., 2011).

While Ts are present in seeds and leaves of most plants, T3 are only present in a small fraction of them (Patacsil *et al*., 2012). In this sense, annatto seeds are the plant source in nature with the highest content of T3 (Aggarwal *et al*., 2010). Ts and T3 are recognized by its antioxidant effects (Patacsil *et al*., 2012). It has been suggested that these compounds act as antioxidants by protecting the lipid membrane from oxidative damage (Aggarwal *et al*., 2010; Frank *et al*., 2012; Lee *et al*., 2012; Quadrana *et al*., 2013). While Ts have been widely studied for their antioxidant properties, there are few studies of T3 on this sense. Several authors have been reported that T3 have an anticancer effect in different kinds of cancer and neuroprotective effect due to its ability to suppress glutamate-induced activation of c-Src kinase, as well

as reducing cholesterol levels (Aggarwal *et al*., 2010). It has been proposed that these beneficial effects might be due to the unsaturation of their aliphatic tail which makes easier their penetration to tissues and confers them a higher antioxidant power (Samarjit *et al*., 2007). In this sense, the characterization of different Mexican accessions of *Bixa orellana* will permit the conservation of the phytogenetic resources, its propagation and the possible establishment of commercial plantations with selected material. The aim of this study was the chemical characterization and quantification of the pigment extraction yield of seven Mexican accessions of *Bixa orellana* and physical color parameters, tocotrienols, tocopherols, norbixin, bixin, total phenolic compounds content and antioxidant capacity in annatto extracts.

# 2 Materials and methods

## *2.1 Achiote seeds samples*

Achiote (*Bixa orellana* L.) seeds were harvested on February 2013 from each of the seven studied accessions from different locations of Merida, Yucatan, Mexico (Table 1). Voucher specimens were authenticated and deposited in Herbarium "U Najil Tikin Xiw" of The Centro de Investigación Científica de Yucatán (CICY), Yucatán, Mexico. Seeds were stored in dark at room temperature until their analysis.

## *2.2 Quantification of the pigment extraction yield*

The pigment extraction yield of annatto was evaluated using three different extraction methods with: 1) a 2% KOH solution (w/v) (alkaline water), 2) absolute ethanol and 3) water, according to the methodologies described by Devia and Saldarriaga (2003). For quantifying the pigment extraction yield (%), 5 g of achiote seeds from each of the studied accessions were used. Color of the crude extracts of 45 mL obtained by the methods mentioned above was measured as described in the following section; then, the extract was placed in a tray and left to stand in the dark at room temperature for 48 h until all liquid evaporated.

The dry pigment was weighted in an analytical weighing scale and the percentage of pigment extraction yield was determined by the following equation:

$$\text{Pigment extraction yield } (\%) = (W2/W1)(100) \quad (1)$$

Where: W2= weight of the obtained achiote pigment and W1= weight of the seeds.

## *2.3 Determination of color parameters*

Forty-five milliliters of each extract above described were used to determine the color parameters luminosity (L*), chromaticity or saturation (C*) and hue (h) using a colorimeter (Hunter Lab, Color Flex, Prufer Comercial Mexico). Each sample was measured in triplicate.

Table 1. Main information on the studied seeds of achiote (*Bixa orellana* L.) Mexican accessions.

| Accession Number | Region | Collection site | GPS coordinates | Voucher number[a] |
|---|---|---|---|---|
| 42 | Yucatán, Mexico | Akil, Akil | N 20°14'57.6"; W 89°20'57.6" | P-Simá-3141 |
| 43 | Yucatán, Mexico | Akil, Akil | N 20°15'35.7"; W 89°20'53.0" | P-Simá-3142 |
| 45 | Yucatán, Mexico | Tekax, Kankab | N 20°11'48.1"; W 89°20'55.5" | P-Simá-3143 |
| 46 | Yucatán, Mexico | Oxkutzcab, Xohuayán | N 20°11'20.0"; W 89°23'00.2" | P-Simá-3144 |
| 47 | Yucatán, Mexico | Oxkutzcab, Xohuayán | N 20°11'10.6"; W 89°23'00.3" | P-Simá-3145 |
| 48 | Yucatán, Mexico | Oxkutzcab, Xohuayán | N 20°11'09.3"; W 89°22'59.8" | P-Simá-3146 |
| 50 | Yucatán, Mexico | Mérida | N 21°00'09.6"; W 89°35'31.9" | G-Godoy-0001 |

[a] Herbarium "U Najil Tikin Xiw" of The Centro de Investigación Científica de Yucatán (CICY), Yucatán, Mexico.

### *2.4 Preparation of the tocopherols and tocotrienols rich fraction (Ts-T3-RF)*

For extracting and purifying tocotrienols and tocopherols from the achiote seeds, 50 mg of seeds from each of the accession were weighted and placed in separate test tubes. Then, the seeds were ground with 4 mL of methanol using an Ultra Turrax high speed homogenizer at 5,000 rpm (Omni International GLH-01, USA) and the obtained samples were vortexed for 1 min. Afterwards, 3.3 mL of chloroform were added to each tube and samples were mixed for 3 min using a vortex; then, 4 mL of Tris-HCl buffer (50 mM, pH 7.5) were added and the sample was mixed again for 1 min using a vortex. Samples were centrifuged at 1,400 g at 4 ºC for 5 min, the organic phase (lower) was recovered in another test tube, the upper phase was discarded and the pellet (intermediate phase) was also recovered for repeating twice the extraction process adding 3.3 mL of chloroform to the pellet. The combined chloroformic phases were diluted to 10 mL with chloroform. Samples were stored in dark at -70 °C until their use (Mène-Saffrané *et al*., 2010). Ts-T3-RF was obtained in triplicate for each studied accession.

### *2.5 Determination of tocotrienols and tocopherols levels by HPLC*

T3 ($\alpha$ and $\delta$) and Ts ($\alpha$, $\beta$, $\gamma$, $\delta$) identification and quantification were performed by high performance liquid chromatography (HPLC) according to the method described by Mène-Saffrané *et al*., (2010). A 200 $\mu$L aliquot of the Ts-T3-RF from each of the studied accessions was taken to dryness under a steam of $N_2$. The dry samples were resuspended with 200 $\mu$L of hexane:methyl tert-butyl ether (90:10 v/v) HPLC grade and filtered through 0.7 $\mu$m glass microfiber (Millipore, USA). The filtered samples were injected into an HPLC system (Agilent Technologies, 1200) consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment and a fluorescence detector. The analyses were carried out on an Agilent Technologies LiChrospher 100 Diol column (5 $\mu$m, 4.6 × 250 mm) using hexane:methyl tert-butyl ether (90:10 v/v) mixture as the mobile phase in an isocratic elution mode. The flow rate was 1 mL $min^{-1}$ and the column temperature was set at 25 ºC. Results were recorded at detector wavelengths for excitation 296 nm and for emission 340 nm. In order to determine the concentration of T3 ($\alpha$-T3 and $\delta$-T3) and Ts ($\alpha$-T, $\beta$-T, $\gamma$-T and $\delta$-T), standard curves (0-5 $\mu$g $mL^{-1}$; and 0-4 $\mu$g $mL^{-1}$ respectively) were prepared using commercial standards (Sigma-Aldrich, USA). Results were expressed as mg $g^{-1}$ seed dry weight (SDW).

### *2.6 Determination of norbixin content*

For quantifying the percentage of norbixin present in achiote seeds, 1g of seeds from each accession was placed in test tubes covered with aluminum foil, 5 mL 0.1 N NaOH were added to each tube and the samples were mixed with a vortex for 2 min. Subsequently, another 5 mL of 0.1 N NaOH were added to each tube and the sample was left to stand for 10 min at room temperature, after which, tubes were shaken for 30 sec using a vortex, the liquid extract was recovered and the seeds were removed. The extract was diluted 1:500 with 0.1 N NaOH and absorbance at 482 nm was measured with a UV-Vis spectrophotometer using 0.1 N NaOH as blank. For norbixin quantification, the specific absorption coefficient ($E^{1\%}_{1cm}$ = 2870) was used (Joint FAO/WHO, 2006). Samples were obtained in triplicate for each studied accession.

### *2.7 Determination of bixin content by HPLC*

Bixin identification and quantification was performed by the method described by Fraser *et al*., (2000) with some modifications. From each of the accession, 50 mg of seeds (in triplicate) were placed in tubes covered with aluminum foil, 5 mL of ethyl acetate were added to each tube and the sample was homogenized with a vortex for 2 min; then, another 5 mL of ethyl acetate were added and the samples were mixed again with a vortex for 2 min. Afterwards, tubes were incubated for 24 h at room temperature and then the liquid extract was obtained and the seeds were removed. All extract were diluted to 10 mL with ethyl acetate and then taken to dryness with a stream of $N_2$. Dry samples were stored at -20 °C until their use.

The dry samples were resuspended with methanol HPLC grade and filtered through 0.45 $\mu$m nylon filters (Millipore Corporation, Bedford, Massachusetts). The filtered samples were injected into an HPLC system (Agilent Technologies 1260) consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment and a MWD (G1315B; Alltech Co. USA). The analyses were carried out on a Waters XTerra MS C18 column (5 $\mu$m, 4.6 × 250 mm) using acetonitrile:methanol:dichloromethane (43:43:14,

v/v/v) mixture as the mobile phase in an isocratic elution mode. The flow rate was 1 mL $min^{-1}$ and the column temperature was set at 25 ºC. Results were recorded at 459 nm with a standard curve of bixin (0 -100 $\mu$g $mL^{-1}$) (Sigma-Aldrich, USA). Results are expressed as mg of bixin $g^{-1}$ seed dry weight (SDW).

### *2.8 Determination of total phenolic compounds and antioxidant capacity*

Three different methods of extraction were used in order to obtain methanolic fractions that could be used to determine the total phenolic compounds and antioxidant capacity in the samples, using whole seeds (A and B extractions) and ground seeds (C extraction) as described below:

A) From each of the accessions, 10 g of whole seeds (in triplicate) were placed in filter paper with a pore size of 25 $\mu$m (Whatman, Germany), extraction cartridges were prepared and placed in the chamber of the Soxhlet equipment. In order to eliminate the oily and fatty material from the samples, three consecutive extractions were performed using 250 mL of hexane (first extraction), ethyl acetate (second extraction) and methanol (third extraction), for 3 h for each extraction. Methanolic fraction (polar) was collected and concentrated to a final volume of 25 mL using a rotary evaporator.

B) A directed extraction with methanol was performed using 10 g of whole seeds (in triplicate) collected from each accession. The samples were placed in filter paper with a pore size of 25 $\mu$m (Whatman, Germany); extraction cartridges were prepared and subsequently placed in the Soxhlet equipment chamber. The extraction was carried out for 3 h (for each sample) using 250 mL of methanol. The polar fraction was collected and concentrated to a final volume of 25 mL using a rotary evaporator.

C) A directed extraction with methanol was performed using 1 g of seeds from each of the accessions (in triplicate). Achiote seeds (1 g) were ground with 25 mL of methanol using an Ultra Turrax high speed homogenizer (Omni International GLH-01, USA). The homogenate was centrifuged at 8,000 g for 10 min at 4 ºC and the supernatant mixed with methanol to a final volume of 25 mL.

All polar fractions were stored in amber flasks at -70 ºC until use.

Total phenolic compounds (TPC) were determined with the Folin-Ciocalteu phenol reagent using the technique described by Singlenton and Rossi (1965). An aliquot of 200 $\mu$L of the adequate dilution (1:5 or 1:10) of each of the polar fractions were mixed with 1 mL of Folin-Ciocalteu reagent [previously diluted 1:10 (v/v) with water] and incubated for 1 min before adding 0.8 mL of 7.5% (w/v) sodium carbonate; then, the reaction mix was incubated for 1 h at room temperature and afterwards, absorbance at 765 nm was measured. In order to determine the concentration of total phenols, a standard curve was prepared using gallic acid (0-100 $\mu$g $mL^{-1}$) (Sigma-Aldrich, USA). Total phenolic compounds were reported as mg gallic acid equivalents per gram of dry seed (mg GAE $g^{-1}$ SDW).

Antioxidant capacity (AC) was determined using the ABTS method as reported by Re *et al*., (1999) with some modifications. For preparing the ABTS solution, 96.2 mg of ABTS [2,2'- Azinobis (3-ethylbenzothiazoline-6-sulfonate] were mixed with 16.5 mg of sodium persulfate in 100 mL of deionized water and the solution was incubated for 16 h in the dark before its use. The ABTS solution was diluted with phosphate buffer solution (PBS, pH 7.4) until an absorbance of 0.7 ± 0.1 at 734 nm was obtained.

Polar fractions obtained as described above were diluted with PBS (1X); 100 $\mu$L of the sample dilution (1:10) were homogenized with 1000 $\mu$L of the diluted ABTS solution and incubated for 10 min at room temperature; afterwards, absorbance at 734 nm was measured using a UV/Visible spectrophotometer. The antioxidant capacity was calculated using a calibration curve with trolox (0-20 $\mu$M) (Sigma-Aldrich, USA). Results were expressed as Trolox Equivalent Antioxidant Capacity per gram of dry seed [TEAC (mM $g^{-1}$ SDW)].

### *2.9 Statistical analysis*

Analysis of variance (ANOVA) followed by the Tukey's multiple means comparison test was performed. The level of statistical significance was P= 0.05. All data were analyzed using the NCSS software (Hintze, 2007).

## 3 Results and discussion

### *3.1 Pigment extraction yield*

Currently, there are several ways for extracting the colorant from *Bixa orellana*. One of the oldest and most traditional methods is the extraction with water (Devia and Saldarriaga, 2003; Taham *et al*., 2015). The efficiency of the pigment extraction

depends on the method (Giridhar *et al*., 2014). In this sense, the general statistical analysis showed that the highest percentage of pigment extraction yields were obtained using KOH (4.84%, in average) as opposed to extractions using ethanol or water (3.46% and 3.37% respectively) according to the Tukey-Kramer Multiple-Comparison Test. Furthermore, no significant differences ($P > 0.05$) in the pigment extraction yield were observed between annatto extracts where ethanol or water had been used. Accessions 50, 43 and 48 showed the highest pigment extraction yields when KOH was used, while no significant differences ($P > 0.05$) were observed among these accessions when ethanol or water were used (Table 2). These results are in agreement with those reported by Devia and Saldarriaga (2003) and Taham *et al*. (2015) where the highest pigment extraction yield was obtained with KOH followed by ethanol and subsequently with water.

## *3.2 Color parameters*

Color is an important characteristic of food. Based on the color of the food, people create expectations whether the food is safe to eat or taste good or not. Based in this idea, the addition of color to food is closely associated with these expectations (Mortensen, 2006). The determination of color parameters in the accessions studied in this work, is important because it allow us to select the possible use of accessions in the industry based in the values obtained from L*, C* and h color parameters as well as the best method of extraction.

In the KOH and water extracts, there were no significant differences ($P > 0.05$) in the values of luminosity (L*), chromaticity (C*) and tone (h) among the accessions (Fig. 1). However, when ethanol was used as solvent, accessions 43 (L*= 4.01 ± 0.79, C*= 7.33 ± 1.07, h= 25.76 ± 6.35) and 50 (L*= 3.17 ± 0.64, C*= 6.81 ± 0.53, h= 26.41 ± 4.41) presented lower values of these parameters compared to the other accessions, which means that these accessions had a darker and redder color (Figs. 1b, 1e and 1h). These results are similar to those reported by Cardarelli *et al*., (2008) who observed that color parameters in annatto extracts depend on the polarity of the solvent used in the extraction procedure; supporting the idea that a lower polarity tend to diminish the values of L*C*h. Alternatively, this behavior could also be explained by the composition of the pigments themselves present in the annatto extracts in ethanol (accessions 43 and 50) where others carotenoids (besides bixin and norbixin) could be extracted, modifying the color parameters obtained (Kopjar and Piližota, 2009).

## *3.3 Content of tocotrienols and tocopherols*

There were significant differences in the content of $\delta$-T3 among the different accessions. Accession 45 showed the lowest levels of $\delta$-T3 (1.72 ± 0.37 mg $g^{-1}$ SDW), while accessions 42 (3.19 ± 0.38 mg $g^{-1}$ SDW), 43 (3.15 ± 0.36 mg $g^{-1}$ SDW) and 48 (3.60 ± 0.50 mg $g^{-1}$ SDW) showed intermediate levels and accession 50 (5.03 ± 0.64 mg $g^{-1}$ SDW) showed the highest level (Fig. 2a).

Table 2. Percentage of pigment extraction yield from annatto extracts.

| Accession Number | KOH (2% w/v) | Ethanol | Water |
|---|---|---|---|
| 42 | $4.38 \pm 0.08^{aA}$ | $3.25 \pm 0.72^{aA}$ | $3.76 \pm 1.23^{aA}$ |
| 43 | $4.68 \pm 0.20^{aB}$ | $3.35 \pm 0.69^{aAB}$ | $2.55 \pm 0.57^{aA}$ |
| 45 | $4.48 \pm 1.03^{aA}$ | $3.38 \pm 0.76^{aA}$ | $3.46 \pm 0.54^{aA}$ |
| 46 | $4.22 \pm 0.08^{aA}$ | $3.13 \pm 0.73^{aA}$ | $3.34 \pm 0.40^{aA}$ |
| 47 | $4.63 \pm 1.19^{aA}$ | $3.02 \pm 0.73^{aA}$ | $2.64 \pm 0.58^{aA}$ |
| 48 | $4.85 \pm 0.15^{aB}$ | $3.27 \pm 0.69^{aA}$ | $4.11 \pm 0.57^{aAB}$ |
| 50 | $6.04 \pm 0.93^{aB}$ | $4.83 \pm 0.62^{aAB}$ | $3.74 \pm 0.64^{aA}$ |

Values are the mean of three replicates ± standard deviation. Same letters, lower case in columns and upper case in rows, do not differ significantly ($p < 0.05$) according to Tukey's test. Abbreviations: KOH, potassium hydroxide; w/v, weight/volume.

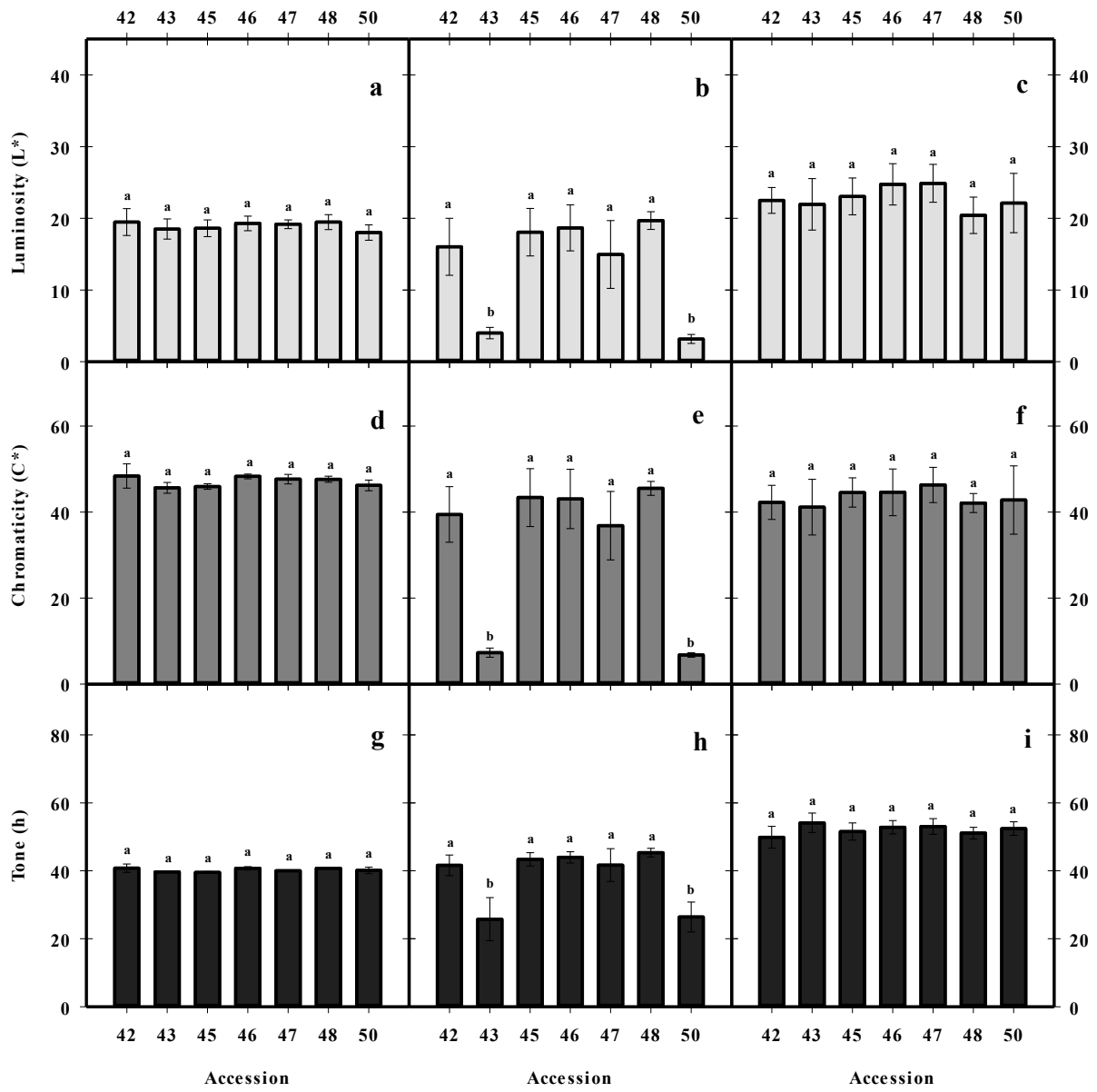


Fig. 1 Color parameters (L*, C*, h) of achiote seeds extracts. Luminosity (L*) in: (a) KOH extracts; (b) ethanolic extracts; (c) water extracts; Chromaticity (C*) in: (d) KOH extracts; (e) ethanolic extracts; (f) water extracts; Tone (h) in: (g) KOH extracts; (h) ethanolic extracts (i) water extracts. Values are the mean of three replicates ± standard deviation. Columns not sharing the same superscript letter are significantly different ($P < 0.05$) according to Tukey's test.

These results are similar to those found by Frega *et al*., (1998) in achiote seeds who reported values of $\delta$-T3 content of 1.40 - 1.47 mg $g^{-1}$ SDW. Currently, it is not known any other plant species with a similar content of T3 ($\delta$-T3, constitutes more than 90%). Plant sources with the highest content of tocotrienols are oils from rice bran, palm and annatto with a T3:Ts ratio of 50:50, 75:25 and 99.9:0.1, respectively (Aggarwal *et al*., 2010). There were not significant differences in the content of $\alpha$-T3 among the different accessions except for accession 50, which showed the highest $\alpha$-T3 content (0.115 ± 0.01 mg $g^{-1}$ SDW) (Fig. 2b).

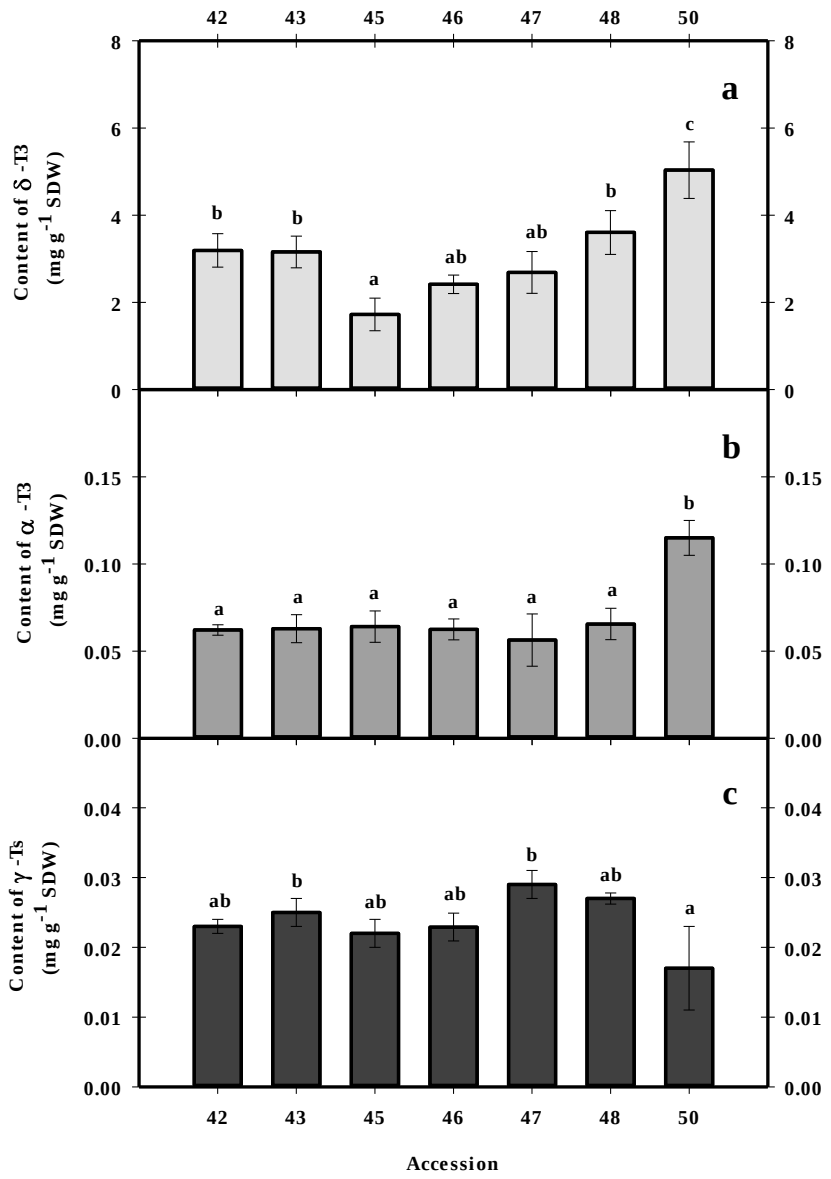


Fig. 2 Content of tocotrienols and tocopherols in seeds of Mexican accessions of achiote (a) $\delta$-tocotrienol, (b) $\alpha$-tocotrienol and (c) $\gamma$-tocopherol. Values are the mean of three replicates ± standard deviation. Columns not sharing the same superscript letter are significantly different ($P < 0.05$) according to Tukey's test. Abbreviations: SDW, Seed Dry Weight; T3, Tocotrienols; Ts, Tocopherols.

Regarding the Ts content, $\gamma$-Ts was the only isoform that was detected in all accessions studied and quantified above the sensitivity limit of the equipment (Fig. 2c). $\alpha$-Ts, $\beta$-Ts and $\delta$-Ts isoforms were found in trace quantities. The lowest content of $\gamma$-Ts was observed in accession 50 and was significantly different to accessions 43 and 47. There were not significant differences among accessions 42, 43, 45, 46, 47 and 48 (Fig. 2c). These results agree with those reported by Frega *et al*., (1998) and Zou and Akoh (2015) who mentioned traces of Ts and consider *Bixa orellana* virtually free of tocopherols. In this sense, figure 3 shows a typical chromatogram of chemical composition of T3 and Ts of a Mexican achiote seeds sample (accession 42). Where Ts were detected in minor amounts compared to the content of T3.

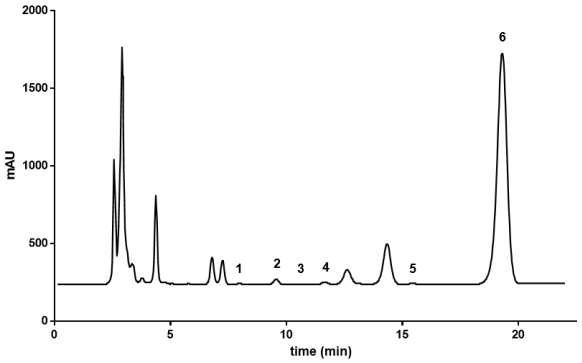


Fig. 3 Representative chromatogram of a fraction of seeds of Mexican achiote from accession 42. The number indicated above a peak represent a compound: 1) $\alpha$-Ts, 2) $\alpha$-T3, 3) $\beta$-Ts, 4) $\gamma$-Ts, 5) $\delta$-Ts, 6) $\delta$-T3. Abbreviations: mAU, milli absorption units.

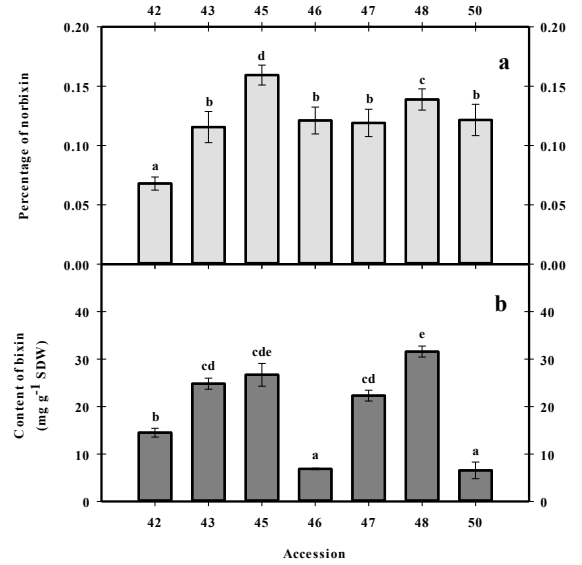


Fig. 4 Content of carotenoids in seeds of Mexican accessions of achiote (a) percentage of norbixin and (b) content of bixin. Values are the mean of three replicates ± standard deviation. Columns not sharing the same superscript letter are significantly different ($P < 0.05$) according to Tukey's test.

## *3.4 Norbixin content*

Carotenoids are natural pigments important for the chemical, food, pharmaceutical and cosmetic industry. These pigments also present an antioxidant capacity attributed to their chemical structure (Mata-Gómez *et al*., 2014). Accession 45 presented the highest levels of norbixin (0.15 ± 0.008%), while accession 42 showed the lowest levels (0.06 ± 0.005%). There were not significant differences ($P > 0.05$) among the other accessions studied (Fig. 4a). These results are similar with those reported by Smith (2006) and Alves *et al*., (2006), who described that norbixin content in *Bixa orellana* aqueous extracts, ranged from 0.1 - 4% and 0 - 3.26% respectively.

## *3.5 Bixin content determined by HPLC*

The content of bixin determined by HPLC showed the highest levels of bixin in accession 48 (31.5 ± 1.16 mg $g^{-1}$ SDW), followed by accessions 45, 43, and 47 (26.6 ± 2.4, 24.8 ± 1.17, 22.3 ± 1.14 mg $g^{-1}$ SDW, respectively) among which there were no significant differences. On the other hand, accessions 46 and 50 had the lowest content of bixin (6.8 ± 0.15 and 6.5 ± 1.74 mg $g^{-1}$ SDW, respectively) with no significant differences between them (Fig. 4b). These results agree with data reported by several authors showing that bixin content in achiote seeds varies from 0.26 to 311 mg $g^{-1}$ SDW (Cardarelli *et al*., 2008; Chisté *et al*., 2011; Rodríguez-Ávila *et al*., 2011). The percentage of bixin is an important factor since it has been established that bixin content on achiote seeds should not be lower than 2.7% for their commercialization in international markets (Giridhar and Parimalan, 2010). Bixin content in annatto seeds may vary between 1 and 4%, depending on the seeds quality (Giridhar *et al*., 2014). In the accession analyzed in the present work, bixin percentage ranged between 0.65 and 3.15% (Fig. 4b). As described above, accessions 48 (3.1%), 45 (2.6%) 43 (2.4%) and 47 (2.2%) showed the highest content of bixin. These results coincide with those reported by Biego *et al*., (2013) who obtained similar bixin percentages in *Bixa orellana* seeds.

On another hand, the fact that accession 50 has shown the lowest content of bixin and a darker and redder color compared to the other accessions might be explained by the copigmentation phenomenon in which there is a molecular interaction between pigments and other organic molecules in solution (referred as copigments or cofactors) causing a more intense coloring of the pigments independently of their concentration (Boulton, 2001). Copigments or cofactors may be flavonoids, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals and anthocyanins (Kopjar and Piližota, 2009). In this regards, Lancaster *et al*., (1994) reported that anthocyanins concentration combined with the interaction of chlorophylls and carotenoids are important factors that determine the red color in apple skin.

Table 3. Total phenolic compounds and antioxidant capacity in polar fractions of achiote seeds.

| Accession Number | WHOLE SEED | | | | GROUND SEED | |
|---|---|---|---|---|---|---|
| | A Polar fraction | | B Polar fraction | | C Polar fraction | |
| | TPC (mg GAE $g^{-1}$ SDW) | AC [TEAC (mM $g^{-1}$ SDW)] | TPC (mg GAE $g^{-1}$ SDW) | AC [TEAC (mM $g^{-1}$ SDW)] | TPC (mg GAE $g^{-1}$ SDW) | AC [TEAC (mM $g^{-1}$ SDW)] |
| 42 | $1.99 \pm 0.22^{bcA}$ | $1.88 \pm 0.09^{cA}$ | $8.36 \pm 0.20^{aB}$ | $5.78 \pm 0.22^{aC}$ | $2.09 \pm 0.05^{aA}$ | $4.31 \pm 0.15^{aB}$ |
| 43 | $1.60 \pm 0.20^{abA}$ | $0.75 \pm 0.03^{aA}$ | $10.05 \pm 0.19^{cC}$ | $7.53 \pm 0.12^{bC}$ | $2.51 \pm 0.04^{cB}$ | $6.28 \pm 0.05^{cB}$ |
| 45 | $4.47 \pm 0.44^{deB}$ | $1.33 \pm 0.09^{bA}$ | $9.64 \pm 0.11^{cC}$ | $16.09 \pm 0.18^{dC}$ | $2.41 \pm 0.02^{cA}$ | $6.21 \pm 0.08^{cB}$ |
| 46 | $4.65 \pm 0.41^{eB}$ | $1.33 \pm 0.04^{bA}$ | $9.10 \pm 0.21^{bC}$ | $8.46 \pm 0.10^{cC}$ | $2.27 \pm 0.05^{bA}$ | $5.84 \pm 0.07^{bB}$ |
| 47 | $1.52 \pm 0.02^{aA}$ | $1.45 \pm 0.07^{bA}$ | $9.67 \pm 0.12^{cC}$ | $7.16 \pm 0.19^{bC}$ | $2.41 \pm 0.03^{cB}$ | $5.55 \pm 0.09^{bB}$ |
| 48 | $4.20 \pm 0.08^{dB}$ | $1.25 \pm 0.02^{bA}$ | $8.19 \pm 0.24^{aC}$ | $8.34 \pm 0.05^{cC}$ | $2.04 \pm 0.06^{aA}$ | $6.31 \pm 0.20^{cB}$ |
| 50 | $2.40 \pm 0.09^{cA}$ | $3.12 \pm 0.04^{dA}$ | $12.06 \pm 0.18^{dC}$ | $16.68 \pm 0.24^{eC}$ | $3.01 \pm 0.04^{dB}$ | $4.54 \pm 0.03^{aB}$ |

[A] Consecutive extraction of polar fraction using soxhlet equipment (starting with hexane (3 h), followed with ethyl acetate (3 h) and finally with metanol (3 h)).
[B] Directed extraction of polar fraction, using soxhlet equipment with metanol (3 h).
[C] Directed extraction of polar fraction, using an ultra turrax high speed homogenizer with methanol.
Values are the mean of three replicates ± standard deviation. Same letters, lower case in columns and upper case in rows, do not differ significantly ($p < 0.05$) according to Tukey's test. Abbreviations: TPC, total phenolic compounds; GAE, gallic acid equivalent; TEAC, trolox equivalent antioxidant capacity; AC, antioxidant capacity; SDW, seed dry weight.

### *3.6 Total phenolic compounds and antioxidant capacity*

Total phenolic compounds (TPC) and antioxidant capacity (AC) are parameters generally determined as part of the phytochemical profile of plant tissues.

There are numerous molecules present in plant-derived natural products that function as antioxidants: carotenoids, phenolic compounds, benzoic acid derivatives, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans and lignins (Pisoschi and Negulescu, 2011). Phenolic compounds, ubiquitous in plants are an essential part of the human diet, and are of considerable interest due to their antioxidant properties (Balasundram *et al*., 2006; Hernández-Carrillo *et al*., 2015).

Concerning the methanolic fractions A, B and C obtained with whole and ground seeds, the polar fractions B showed the highest ($P < 0.05$) content of TPC in comparision with polar fractions A and C. However, the methanolic fractions B obtained with soxhlet extractor were more efficient in general, producing a significant increase of 4.0 and 3.3 fold the extraction of TPC, in comparision with methanolic fraction C obtained with ultra turrax and polar fraction A (consecutive extraction), respectively. The TPC content in the methanolic fractions B and C showed a similar behavior in all the accessions analized. Accession 50 showed the highest content of TPC in both polar fractions B and C (12.06 ± 0.18; 3.01 ± 0.04, respectively); followed by accession 43, 45, 46 and 47. Finally, the lowest TPC was observed in accessions 42 and 48 (Table 3). The results obtained in the present work regarding the TPC in the polar fractions analyzed agree with those reported by Cardarelli *et al*., (2008) and Giorgi *et al*., (2013), who observed that TPC in *Bixa orellana* extracts ranged from 0.30 to 4.67mg $g^{-1}$ SDW.

On other hand, the evaluation of the AC may be an appropriate tool to determine the additive antioxidant properties of plant-derived natural products. The ABTS is a rapid, simple, accurate, inexpensive and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate the antioxidant capacity of foods. However, AC by ABTS has some limitations, because the validated method is directed to polar compounds. In recent years there has been increased the interest in evaluate the antioxidant potential of plants, seed extracts and bioactive compounds and their possible application as functional foods and nutraceuticals (Morales-Delgado, 2014; Martínez-Palma *et al*., 2015; Gómez-Sampedro and Zapata-Montoya, 2016).

The AC in the polar fractions showed a similar trend to the one observed for TPC. The polar fractions B showed the highest AC in all the accessions studied, followed by polar fractions C and the lowest AC in polar fractions A. The AC (TEAC mM $g^{-1}$ SDW) in polar fractions B showed a significant increase of 1.79 and 6.32 fold, in comparision with the polar fractions C and A, respectively (Table 3). The results obtained are similar to those reported by Cardarelli *et al*., (2008) who observed a similar AC in achiote extracts obtained with polar solvents.

# Conclusions

The chemical characterization of Mexican accessions of achiote showed that four of the seven studied accessions have the potential to compete in international markets due to their bixin content. Furthermore, chemical characterization of different accessions will permit the selection of commercially relevant phytogenetic resources, and the possible establishment of large-scale plantations with selected accessions. The results of this study demonstrated that the highest content of a commercially valuable metabolite (such as bixin) does not necessarily correlate with the highest content of other important metabolites (tocotrienols). In this sense, accession 50 which had a lower content of bixin (6.5 ± 1.74 mg $g^{-1}$ SDW) showed the highest content of tocotrienols $\delta$-T3 (5.035 ± 0.64 mg $g^{-1}$ SDW), since these two metabolites share a common intermediate (geranyl geranyl diphosphate) in their biosynthetic pathway and there is a differential regulation for the synthesis of one or the other metabolite. Also, it is important to note that the accession 50 exhibited the highest TPC and AC, in addition to the highest content of T3, mainly in the isoform $\delta$-T3. The results presented here should therefore be considered in order to determine the most adequate use of the characterized phytogenetic resources.

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## Notation

| | |
|---|---|
| L* | luminosity |
| C* | chromaticity |
| h | tone |
| $\alpha$ | alpha |
| $\beta$ | beta |
| $\gamma$ | gamma |
| $\delta$ | delta |
| T3 | tocotrienol |
| Ts | tocopherol |
| Ts-T3-RF | tocopherols and tocotrienols rich fraction |
| ABTS | 2,2'- Azinobis (3-ethylbenzothiazoline-6-sulfonate) |
| SDW | seed dry weight |
| GAE | gallic acid equivalents |
| TPC | total phenolic compounds |
| TEAC | trolox equivalent antioxidant capacity |
| AC | antioxidant capacity |

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