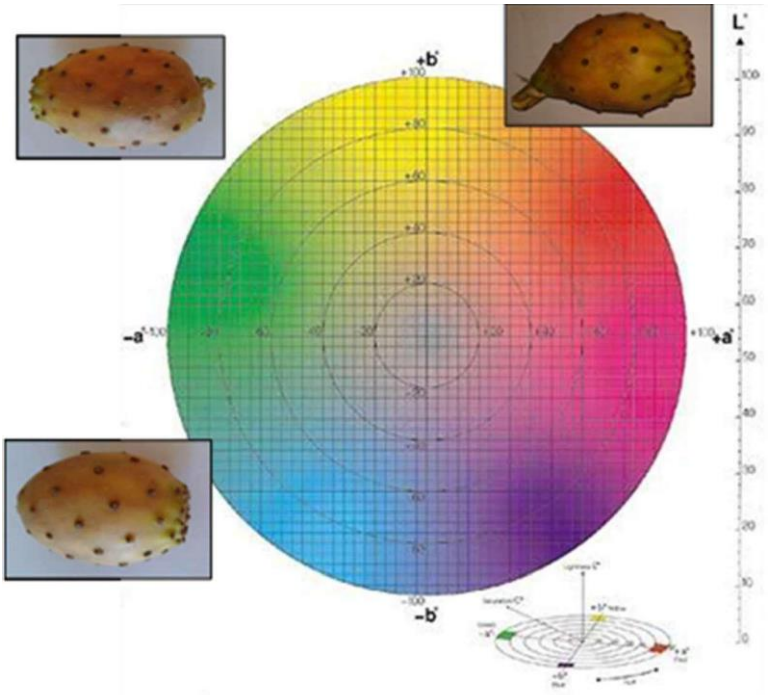

Table of contents graphic. Study of CIELAB colour space (a*b*)-plane on different parts of the three varieties of Opuntia-ficus indica.
138x127mm (72 x 72 DPI)

Betalain profile, phenolic content and color characterization of different parts and varieties of *Opuntia ficus-indica*

María Jesús Cejudo-Bastante[a], Makhlouf Chaalal[b], Hayette Louaileche[b], Juan Parrado[c], Francisco J. Heredia[a]*

[a] Food Color and Quality Lab., Dept. Nutrition and Food Science, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain

[b] Laboratoire de Biochimie Appliquée, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000, Bejaia, Algérie

[c] Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Seville, 41012, Seville, Spain

María Jesús Cejudo-Bastante: mjcejudo@us.es

Makhlouf Chaalal: makhlouf.chaalal@yahoo.fr

Hayette Louaileche: haylouaileche@yahoo.fr

Juan Parrado: parrado@us.es

Francisco J. Heredia: heredia@us.es

Corresponding author:

Tel.: +34 954556495 Fax: +34 954556110

e-mail: heredia@us.es

ABSTRACT

Three different varieties of *Opuntia-ficus indica* (*R*, red; *Y*, yellow; *RY*, red-yellow) have been considered in this study. Our attention was focused on differential tristimulus colorimetry, and on the analysis of individual betalains (HPLC-DAD-ESI-ToF-MS) and phenolic content, scarcely previously reported in that kind of samples. The importance of this research stems from the elucidation of the parts and varieties of cactus pear more optimal to be used as natural colorant and source of phenolics and betalains. Thus, the *RY* pulp was appropriate to obtain colorants with high color intensity (C^*_{ab} = 66.5), whereas the whole *Y* fruit and *R* pulp reached a powerful and stable yellow and red color, respectively (C^*_{ab}/h_{ab}: 57.1/84.7 and 61.1°/81.8°). This choice was also based on the visually appreciable differences ($\Delta E^*_{ab}>5$) among samples, mainly quantitative ($\%\Delta^2L$, $\%\Delta^2C$). In addition, seeds of all *Opuntia* varieties showed significantly ($p<0.05$) similar phenolic content (around 23.3 mg/g) and color characteristics.

Keywords: *Opuntia-ficus indica*, differential tristimulus colorimetry, betalains, phenolics, HPLC-DAD-ESI-ToF-MS.

INTRODUCTION

In the last few years, consumers are more conscious about the importance of the healthy and natural food both in the manufacture or consumption. One part of this reality is the use of natural products as colorants. In this way, there is a large extent of natural colorants, extracted from different natural raw materials, rich in compounds such as anthocyanins, carotenoids and chlorophylls. Apart from those, there is a group of chemical compounds that it is still poorly-investigated, betalains.

Betalains are water-soluble compounds present in a restricted number of families of the plant order Caryophyllales and of the genus *Amanita* of the Basidiomycetes [1]. Numerous advantageous on consuming betalain-rich food have been reported, such as antioxidant activity, antiviral, anti-inflammatory and anti-carcinogenic effects [2, 3]. Betalains are structured in two chemical families (betacyanins and betaxanthin). The common moiety of both chemical families corresponds to betalamic acid, and the nature of the addition residue determines the pigment classification as betacyanin (hydroxycinnamic acid derivatives or sugars) or betaxanthin (amines or amino acids) [4], with maximum absorptions around 540 nm and 480 nm, respectively [2, 5].

There are manifold species and varieties used as natural food colorants. In that way, although the most studied and widely distributed source of betalains is the red beet (*Beta vulgaris* L.) [6-8], betalains have been also studied in other raw materials. Some examples are the Swiss chard vegetable [9], flowers (*Gomphrena globosa* L. and *Bougainvillea* sp.) [10], and fruits such as pitaya [11] and cactus pear (*Opuntia* sp.) [12-16].

Opuntia ficus-indica is a type of cactus that provides edible prickly pears. This kind of cactus is widely cultivated in the world due to its adaptability to hard growing conditions (arid and semi-arid zones) [17]. Recently, the study of cactus pear is becoming more and more prominent because of their high content of betalains and other phenolic

compounds. In fact, those compounds have been previously studied on this matter in different edible and non-edible parts of prickly pears such as pulps [14], skins [16] and seeds [18, 19]. These researches have been focused on the general chemical characterization [12, 20], the total content of both phenolic and betalains [21-23] and the antioxidant activity determination [24]. However, only a few researches have been developed about individual betalains in that raw material [13, 14, 25] Moreover, although this fruit has a potential enterprise projection as natural colorant, hitherto, scarce advanced colorimetric studies have been developed [12, 21, 26] Therefore, there are still many aspects to be elucidated about the repercussion on the color of *Opuntia-ficus indica* when it was added as safe colorant.

Therefore, the aim of this research was to carry out an extensive chemical and colorimetric characterization of *Opuntia-ficus indica* [L.] Mill cultivated in Algeria. Concretely, different parts of three cactus pear varieties were taken into account, which have not been previously considered in conjunction. Our interest was focused on the study of the phenolic content and individual betalain profile, and also on colorimetric characteristics by applying differential tristimulus colorimetry, scarcely reported in literature. Therefore, the objective is not only the chemical characterization of different parts of prickly pears, but its practical application as natural colorants by deepening on the study of differential colorimetry and betalain profile.

MATERIALS AND METHODS

Chemical and Solvents

Methanol of analytical or HPLC grade were purchased from J. T. Baker (Baker Mallinckrodt, Mexico), and formic acid and Folin-Ciocalteau reagent were supplied by Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water was obtained by a Milli-Q plus water purification system (Millipore Corp., Bedford, MA). Sodium ascorbate and

L-ascorbic acid were from Panreac (Barcelona, Spain). Betanin was supplied by Cymit Quimica S.L. (Barcelona, Spain).

Samples

Three different cultivars of prickly pear fruits (*Opuntia ficus-indica* [L.] Mill.) with diverse physical qualities have been selected for this study. Those cactus pear varieties were typically cultivated in the area of Bousselam (Setif, Algeria). Fully ripe cactus pears were collected in August in different points of the plant and in various parts of the parcel. Samples were selected on the basis of their color (both pulp and skin), shape and presence of cladode spines (*R*, red, ovoid and cladode spineless; *Y*, yellow, elongate with cladode spines; *RY*, red-yellow, ovoid with cladode spines). Fruits were harvested with a desiderable maturity and in good sanitary conditions (*R*, *Y* and *RY*: pH, 6.14, 5.86 and 5.95; titratable acidity, 0.10, 0.11 and 0.09; °Brix, 12.83, 11.55 and 14.22, respectively).

Fruits were carefully washed and manually peeled. The seeds were removed from the pulp and washed with distilled water. Three different parts of each variety was studied, corresponding to the edible part of the fruit: seeds, pulp, and the whole fruit (seeds+pulp). They were separately lyophilized (Christ, Alpha 1-4 LD plus, Germany), ground with a crusher (IKA A 11B, Germany), and passed through a 500 µm sieve.

Preparation of extracts

Lyophilized samples (1 g) were added to 3 mL of methanol: water (50:50) containing 50 mM of sodium ascorbate (for avoiding possible oxidations). Subsequently, samples were stirred at 225 rpm for 10 min in darkness. Afterwards, samples were centrifuged at 12000 x *g* at 10 °C for 5 min, separating supernatants from the plant tissue. In order to achieve the complete discoloration of the plant material, the residues was rinsed once more with the extraction solution and finally with 100% methanol. The extracts were

then concentrated in vacuum (30 ºC) until around 3 mL in order to remove methanol, adding purified water until a total of 4 mL. The mean pH values of the resulting extracts were similar among varieties, as follows: seeds, 5.83; pulp, 6.20; and the whole fruit, 6.04. All experiments were carried out in triplicate.

Colorimetric measurements

A Hewlett-Packard UV-vis HP8452 spectrophotometer (Palo Alto, CA) was used to carry out color measurements. The whole visible spectrum (380-770 nm) was recorded at constant intervals ($\Delta\lambda$=2 nm) using 2 mm path length glass cells and distilled water as reference. The CIELAB parameters (L^*, a^*, b^*, C^*_{ab}, and h_{ab}) were determined by using the original software CromaLab© [27] following the Commission Internationale de L'Eclariage's recommendations [28]: the CIE 1976 10° Standard Observer and the Standard Illuminant D65. Euclidean distance between two points in the three-dimensional space define by L^*, a^*, and b^* were used for calculating color differences (ΔE^*_{ab} of CIELAB): $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Moreover, the relative contribution of the three color attributes respect $\Delta^2 E^*_{ab}$ that constitute the total CIELAB color difference was also calculated, expressed as percentage of the quadratic increases of lightness, chroma and hue [29].

Total phenolic content

Total phenolic content was determined using a modification of the Folin-Ciocalteau method [30]. Absorbance was measured at 765 nm, and the results were expressed as milligrams of gallic acid per liter (mg GAE/L). Subsequently, total phenolic content was expressed as mg/g of dry weight (DW). In order to eliminate the contribution of the ascorbic acid to the measurement of absorbance in the Folin-Ciocalteau assay, a correction factor was calculated [31], quantifying also the ascorbic acid present in each

sample [32] Subsequently, the impact of ascorbic acid in terms of mg GAE/L were deducted from the spectrophotometrically determined total polyphenol values.

HPLC-DAD-ESI-ToF-MS analysis of betalains

HPLC separation, identification and semi-quantification of betalains were performed in an Agilent 1200 chromatographic system equipped with a quaternary pump, an UV–vis diode-array detector, an automatic injector, and ChemStation software (Palo Alto, CA, USA). Prior direct injection, the samples were filtered through a 0.45 µm nylon filter (E0034, Análisis Vínicos, Spain). All analyzes were made in triplicate.

The betalains identification was carried out following the method proposed by Castellanos-Santiago and Yahia [14], using 1% formic acid in water (v/v, eluent A) and methanol (eluent B). Betalains were separated in a Zorbax C18 column (250 x 4.6 mm, 5 µm particle size) maintained at 25 °C, at a flow rate of 1 mL/min. The injection of the volume for all extracts was 20 µL. Betalain compounds were separated starting with 100% A, followed by a linear gradient from 0% B to 10% B in 20 min, then a linear gradient from 10% B to 30% B in 10 min, and from 30% B to 100% B in 5 min. In order to re-establish the initial conditions, a linear gradient from 100% B to 100% A was used during 5 min. UV–Vis spectra were recorded from 200 to 800 nm with a bandwidth of 1.0 nm. Betacyanins and betaxanthins were monitored at 535 and 482 nm, respectively. The identification of each chromatographic peak were tentatively assigned by their visible spectral characteristics in comparison with standard and retention times according to the method proposed by Castellanos-Santiago and Yahia [14] Semi-quantification was done on the basis of the mean areas of individual betalains.

The identity of individual betalains was confirmed by mass spectrometer. The separation was performed in a Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA), using the column and mobile phase above indicated

but with a post-column split of 0.4 mL/min. The mass spectra was obtained using a microToF-QII High Resolution Time-of-Flight mass spectrometer (UHR-ToF) with Q-ToF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. The instrument was operated in positive ion mode using a scan range from *m/z* 50-1200. Nitrogen was used as the dry gas at a flow rate of 8 mL/min with nebulizing (1.2 bar), and nebulized temperature was set at 200 °C. Mass spectra were acquired in MS full scan mode and data were used to perform multi target-screening using Target Analysis™ 1.2 software (Bruker Daltonics, Bremen, Germany). The instrument control was performed using Bruker Daltonics HyStar 3.2. The accurate mass data of the molecular ions were processed through the software Data Analysis (Bruker Daltonics, Bremen, Germany). The accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm.

Statistical analysis

Statistical analysis was carried out by using Statistica version 8.0 software [33]. Univariate analyses of variance (Tukey test and ANOVA) and correlation analysis were applied to discriminate among the means of chemical data by 'variety' and by 'part of the fruit' factors.

RESULTS AND DISCUSSION

Colorimetric characteristics

The color was objectively calculated by tristimulus colorimetry, and the values of L^*, C^*_{ab} and h_{ab} of each sample are shown in Table 1. The color points represented in the (a^*b^*)-color diagram as well as the lightness of the sample extracts appeared in Fig. 1. The different locations of the diverse parts of *Opuntia* varieties could permit to establish objectively the chromatic characteristics of the fruits. As it can be seen, two different groups are formed: seeds (S) on the one hand, and pulp (P) and the whole fruit (W), on

the other hand. Regardless of the 'variety', P and W were located in the first quadrant (positive values of a^* and b^*) of the (a^*-b^*)-plane, whereas S were situated between the first and second quadrant (positive values of b^*). It can be noticed that, although seed extracts showed intense yellowish tonalities (values of hue close to 90°), their chromatic intensity was very low (values of C^*_{ab} between 4 and 8) (Table 1). However, P and W had a very intense orange-yellow color (values of C^*_{ab} and h_{ab} around 60° and 82°, respectively) (Table 1). Pulp extracts of the different varieties were clearly separated in the space according to both b^* and a^* parameters, whereas the whole fruits were discriminated only on the basis of b^* (Fig. 1). Moreover, a separation 'by variety' of these samples was observed (Fig. 1). Thus, red-yellow (*RY*) variety showed the highest values of b^*, whereas a^* is the predominant parameter in red (*R*) variety. With regard to the lightness, seeds and pulp (especially *R*P) was noticed to occupy opposite positions, keeping the whole fruit extracts in an intermediate stage.

Among 'variety' and 'part of the fruit' factors, it was largely the latter the most influent factor affected on color (Table 1). When ANOVA was applied to the set of data in order to draw attention to the possible significant differences according 'variety' and 'part of the fruit' (n=27 and n=9, respectively; data not shown), only the latter factor showed significance. In order to a better understanding, a comparison by pairs using Tukey test was applied (Table 1). Thus, when considering the 'part of the fruit' factor, the major significant variations on CIELAB parameters were assigned to *R* variety. Clearly, as it could be expected, S significantly differed from P and W for all *Opuntia* varieties in all colorimetric parameters. Moreover, due to the absence of significance ($p > 0.05$) in C^*_{ab} and h_{ab} between P and W extracts in *Y* and *RY* varieties, both pulp or the whole fruit for both varieties could lend the same color to foods and they could be used indistinctly as natural yellow colorant. However, the values of chroma in P extracts of *R* variety were

significantly higher when comparing with W, with a considerably strong red tonality. Seeds could contribute to those differences between W and P because of their low values of chroma, producing an attenuation of the color intensity of W (Table 1). Therefore, it is more suitable to use the pulp of *R* variety for conferring a more powerful red color.

With regard to the 'variety' factor, P was considered the most influent part of the fruit to discriminate among varieties, above all regarding to chroma and hue. Thus, if pulp extract was considered to be used as natural colorant, *Y* variety would confer the significantly highest yellow tonalities [34]. On the contrary, if the whole fruit extract was taken into account, *RY* variety should be employed, due to its noticeably higher chromatic intensity. It was also remarkable that the colorimetric characteristics were not significantly affected by 'variety' when S extracts were taken into account.

From an industrial point of view, it is easier and economically advantageous to avoid the time-consuming manipulation that some technological procedures takes, such as the separation of the seeds from the pulps. Therefore, the whole *Y* fruit and the pulp of *R* varieties are optimal to be used as yellow and red colorant, respectively, whereas the whole *RY* fruit could be useful as additive to confer vivid colors.

Differential colorimetry

In an attempt to evaluate the colorimetric differences among 'varieties' and 'parts of the fruit', the mean color difference (ΔE^*_{ab}) among samples were calculated (Fig. 2). Taking into account that ΔE^*_{ab} of up to 5 CIELAB units indicates 'big color differences' appreciable to the human eyes [35, 36], it was confirmed that visually perceptible color differences were observed among the different parts of the fruit of each variety (Fig. 2a). On the one hand, the significant variations among S/P and S/W for all varieties previously commented were also appreciable to human eyes. This fact is

predictable because they greatly differ from phenolic profile and, consequently, from the color [34]. When the roles of each color attribute respect $\Delta^2E^*_{ab}$ was calculated ($\%\Delta^2L$, $\%\Delta^2C$, $\%\Delta^2H$), results evidenced that these differences were mainly quantitative, with major values of quadratic variations of chroma ($\%\Delta^2C > 90$), and practically negligible of lightness and hue. In addition, also noticeable and quantitative color differences experimented W and P in *R* variety, being lightness the main responsible ($\%\Delta^2L > 65$). The negligible quadratic percentages of hue demonstrated that this parameter did not contribute to establish significant differences of color.

On the other hand, when a comparison among varieties was carried out (Fig. 2b), it was observed that the several significant differences in the pulps were mainly due to chroma ($\%\Delta^2C$~65 and 87 when comparing *Y*/*R* and *Y*/*RY*, respectively). However, it was lightness the main responsible of the discrepancy between *R*/*RY* varieties ($\%\Delta^2L = 68$). In the case of the whole fruit, only quantitative variations contributed to the color differences among *RY*/*Y* and *R*/*RY* ($\%\Delta^2C > 95$). The contribution of quadratic variations of lightness was negligible. No appreciable color differences among varieties were found in seeds.

Polyphenolic content

The values of phenolic compounds of the different parts of cactus pears were shown in Table 1. ANOVA test applied to the whole set of data revealed that no significant differences were assigned either by 'part of the fruit' or by 'variety' (data not shown). However, as it was performed with CIELAB parameters, Tukey test was applied in order to elucidate possible significant variations by pairs. It is highlighted that the 'part of the fruit' factor showed significance for all three varieties (Table 1). In the case of *Y* both varieties could lend the same color to foods and they could be used indistinctly as variety, as it was expected, the phenolic content of W was the most predominant, natural yellow colorant. However, the values of chroma in P extracts of *R* variety were followed by P and S. Khatabi et al. [34] also reported abundant amount of polyphenols in

the whole fruit and pulp of Moroccan prickly pears. Furthermore, according to the 'variety' factor, *Y* variety (both P and S) had the significantly lowest phenolic content in comparison with the rest of varieties. Similar results were also obtained by Khatabi et al. [34] when comparing pulps of yellow and red varieties of prickly pears. Furthermore, seeds were attributed to the minor values of total polyphenols, but, even so, prickly pear seeds could be considered as an important natural source of polyphenols with direct application in food or health industry [18, 19]. Any variance on the polyphenolic content among varieties was found, contrarily to that observed by Morales et al. [22] in seeds of diverse species of *Opuntia*.

Betalain identification

In this research, several types of betalains have been identified by HPLC coupled with electrospray mass spectrometry. The identities of betalains were achieved on the basis of the retention times, spectra and *m/z* values. Figure 3 shows the chromatographic pattern and peak assignment of the two families of betalains (betacyanins and betaxanthin), monitored at 535 and 482 nm, respectively. As well, the EIC chromatogram and MS positive mode of the major compounds belonged to each kind of betalains (betanin and indicaxanthin, respectively) were included. These compounds are summarized in Table 2, which shows the retention times, UV-Vis data and mass spectral of each individual betalain identified of *Opuntia-ficus indica* by HPLC-DAD-ESI-ToF-MS. Concretely, the whole fruit of red-yellow variety has been selected as representative because of their higher values of areas. Individual betalains were identified in both pulp and in the whole fruit. However, seeds lacked betalains.

A large extent of betaxanthins has been identified in this study. Among them, the presence of indicaxanthin, well-described in cactus pears, has been confirmed (peak 4 both varieties could lend the same color to foods as they could be used indistinctly as natural yellow colorant. However, the values of betanin in extracts of R variety were; molecular ion at 309.1102 *m/z* units, and a daughter ion at *m/z* 263.1018) (Table 2;

Figure 3). Moreover, it is highlighted the identification of several additional betaxanthins, whose structure involved amino acids such as amino butyric acid (peak 3, *m/z* 297.1106 and 253.1227), valine (peak 5, *m/z* 311.1275 and 137.0628), isovaline (peak 6, *m/z* 311.1195 and 137.0654), isoleucine (peak 7, *m/z* 325.1408 ad 219.1094) and leucine (peak 8, *m/z* 325.1369 and 209.0844) (Table 2). Other betalains have been tentatively identified, on the basis of UV-vis spectra and the retention times of the chromatographic method used [14]: muscaarin (peak 1, 476 nm), vulgaxanthin (peak 2, 472 nm) and phenylalanine (peak 9, 468 nm). In addition, three betacyanin compounds have been correctly assigned: the well-known betanin (peak 10, *m/z* 551.1554), its isomer isobetanin (peak 11, *m/z* 551.1485) and a minor one, poorly described, called gomphrenin (peak 12, *m/z* 551.1987). The different attachment of the glucose moiety (C-6 and C-5) caused the different elution times between gomphrenin and betanin, respectively [14] All betacyanins produced a fragment near of 389.1243 *m/z* units corresponding to the protonate aglycones ([betanidin + H]$^+$ or [isobetanidin+H]$^+$) (loss of a fragment of *m/z* 162 units corresponding to the glucose molecule).

Semi-quantification of betalain

Two different wavelenghts have been monitored in order to carry out the semi-quantification of betalains, 535 nm for betacyanins and 482 nm for betaxanthins. It is noteworthy that, although both betacyanins and betaxanthins were present in all cactus pear varieties, the mean areas of betaxanthins were, by far, considerably higher in comparison with that of betacyanins (Table 3). Among them, indicaxanthin was the major betaxanthin compound in *Opuntia-ficus indica*, with an average percentage of area around 90%. Similar results were reported in the pulp of different Mexican and