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Characterization of carotenoid profile of Spanish *Sanguinos* and *Verdal* prickly pear (*Opuntia ficus-indica*, *spp.*) tissues

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ABSTRACT

Carotenoid profiles of different tissues (peel, pulp and whole fruit) of Spanish *Sanguinos* (red) and *Verdal* (orange) prickly pears (*Opuntia ficus-indica spp.*) have been characterized in detail and quantified for the first time. Carotenoids were determined by HPLC-PDA-MS (APCI$^+$), using a reverse phase C_{30} column. A total of 9 xantophylls and 4 hydrocarbon carotenes were identified. Also, minor amounts of chlorophyll a, a′ and b can be observed in *Opuntia* peel extracts. All carotenoids were found to be present in their free form (no carotenoid esters were detected). The RAE was highest in *Opuntia* peels, showing values from 19.20 to 16.48 μg/100 g fresh weigth, for *Sanguinos* and *Verdal Opuntia* fruits, respectively. The main carotenoid in *Opuntia* peel extracts was (all-E)-lutein with 1132.51 and 767.98 μg/100 g fresh weigth, followed by (all-E)-β-carotene with 200.40 and 173.50 μg/100 g fresh weigth for *Sanguinos* and *Verdal* varieties of *Opuntia* fruits, respectively.

1. Introduction

Due to their high fiber, mineral and antioxidant content, prickly pear fruits (*Opuntia spp.*) are ideal sources for the development of nutraceuticals or functional ingredients. Prickly pear fruits belong to the *Cactacea* family which includes more than 200 species which grow abundantly in the arid regions of the world in countries such as in Mexico, United States, Spain and Italy, among others. Prickly pear fruit is widely employed in Latin America for the preparation

Abbreviations: HPLC-DAD, high-performance liquid chromatography- diode array detection; UV–Vis, ultraviolet–visible; APCI-MS, atmospheric pressure chemical ionization- mass spectrometry; RAE, retinol activity equivalents; DPPH, ·2,2-diphenyl-1-picrylhydrazy; ORAC, oxygen radical absorbance capacity.

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of juices, nectars, syrups, marmalades, and other food products, meanwhile the current demand of prickly pears in Spain is on the rise. There is a big area of opportunity for the revaluation of waste products (such as prickly pear peels) derived from these industries. These waste products could be used for carotenoid extraction or direct incorporation in other food matrixes as a functional ingredient.

Carotenoids belong to the isoprenoid group and are widely distributed among intense colored fruits where they contribute to the fruit's appearance and attractiveness. These pigments are responsible for most yellow, orange and red colors in vegetables. They possess a high nutritional value due to the fact that they can act as dietary antioxidants where some are provitamin A carotenoids such as β-carotene present in carrots, and β-criptoxanthin present in oranges and papayas (de Ancos, Gonzalez, & Cano, 2000). Until now, little information regarding the carotenoid profile in prickly pears has been reported, which remains of great interest in the characterization of these fruits. However, prickly pear total carotenoid content has been reported in the literature (Fernández-López et al., 2010). Mondragon-Jacobo, Yahia, and Castellanos (2009) reported the presence of some carotenoid species found in 10 mexican prickly pear cultivars where they identified traces of neoxantin, violaxantin and lutein.

Carotenoids play an important role in the prevention of human diseases and maintaining good health as part of a balanced diet. Although carotenoids' main action mechanism is attributed to their antioxidant capacity, their complete physiological effects are not completely explained since carotenoids form part of a complex metabolism. Through a synergistic cooperation with other antioxidants they may act for the benefit of some food patterns such as the Mediterranean diet (Gammone, Riccioni, & D'Orazio, 2015). The main carotenoid found in prickly pear, lutein, has shown to prevent lipid peroxidation due to its reactive oxygen species (ROS) scavenger capacity, therefore proving to exert a protective role against age-related macular degeneration (AMD) and senile cataract (Chiu & Taylor, 2010). β-carotene, the most widely studied carotenoid which is also present in prickly pears, is a prehormone that is converted into retinoic acid, which functions as a ligand through the regulation of expression of genes involved in the metabolic process (Ross, Zolfaghari, & Weisz, 2001). Zeaxanthin is found, along with lutein, in the macula lutea and is implied in the health of the eye, although both may also be involved in cardiovascular aspects which result inversely correlated with carotid artery stiffness, pulse wave velocity and elastic modulus (Gammone et al., 2015). Another carotenoid found in the red varieties of prickly pear, lycopene, has been found to prevent aging and cardiovascular diseases by eliminating reactive oxygen species (ROS), to inhibit lipid peroxidation and reinforce the immune system (Omoni & Aluko, 2005). Lycopene has also been reported to reduce the risk of several types of cancer such as prostate, breast, lung and digestive tract (Wu et al., 2003). In order to be able to exert their specific health benefit in the human body, carotenoids must first be ingested, released from the food matrix and dispersed in the gastrointestinal tract. Carotenoids must consequentially be solubilized in micelles consisting of phospholipids, free fatty acids, monoacylglycerols and bile salts. Carotenoid solubilization is a key step leading to the uptake by intestinal cells and therefore conditions bioaccesibility (Yonekura & Nagao, 2007).

Prickly pear composition regarding compounds other than carotenoids (vitamin C, betalains, total phenolic compounds and flavonoid content) has been widely studied. Ferulic acid has emerged as principal phenolic compound, meanwhile the flavonoid fraction consists mainly of rutin and isorhamnetin derivatives which are responsible for antioxidant activity. Several authors have compared prickly pear seeds and pulp regarding fatty acids, lipids, sterols, liposoluble vitamins and β-carotene where a high quantity of neutral lipids were observed in seed oil, meanwhile the pulp oil was found to be richer in glucolipids and phospholipids (Ramadan & Mörsel, 2003). Betalains are also important phytochemical compounds in prickly pear fruits and contribute to antioxidant activity. Betalains are hidrosoluble nitrogenated pigments synthesized from tyrosine and include two classes of compounds: betacyanins and betaxantins. The characterization of the betalain pigments in prickly pear fruits was reported by Fernández-López and Almela (2001). In prickly pear, vitamin C is the third main vitamin and possesses antioxidant properties. Several values of vitamin C content have been reported in the literature, which are dependent on the analyzed prickly pear variety.

As mentioned earlier, some studies have been able to identify some of specific carotenoids in prickly pear but not all (Yahia, Castellanos, & Mondragon-Jacobo, 2010). However in the present study, the quali- and quantitative composition of carotenoids in different the prickly fruit tissues. The objective of this study is to describe the complete carotenoid profile present in Spanish prickly pears (*Opuntia ficus-indica*), yellow and red skinned cultivars. Also, the quantification carotenoid species present in different tissues of prickly pear (whole fruit, pulp and peel) is done. Complementary information of other constituents these fruits is showed and the correlation of each chemical compound with the antioxidant activities (DPPH and ORAC assays). This study may lead the basic knowledge to the development of future functional foods and functional ingredients or nutraceuticals with potential health benefits from *Opuntia* tissues.

2. Materials and methods

2.1. Prickly pear fruits

Fruit collection was done at the beginning of the ripening period, at 85% color break stage (skin coloring). This study was limited to two Opuntia ficus-indica Spanish cultivars namely: *Sanguinos* (red) and *Verdal* (orange) from a commercial orchard Bioarchen® located in Archena (Murcia, Spain; 38°7′N, 1°18′W; 121 m over sea level). The characteristics of the ripening state of prickly pear fruits are showed in Table 1. These fruits were hand-picked, washed and processed manually for the separation of their respective tissues: peel, pulp and whole fruit. Their physicochemical characteristics such as total fruit weight (g), apical caliber (cm) and equatorial caliber (cm) were measured directly in ten whole fruits for each variety. Other characteristics as pH and soluble solids (as Brix degrees at 25 °C) were measured from juice obtained from the prickly pear pulps. Samples from peel, pulp and whole fruit of each variety were frozen with liquid nitrogen, and freeze-dried (Telstar LyoBeta 15®). Seeds were removed before pulverization in a cutter mill (Grindomix de Retsch GM200®) and stored at −20 °C until the time of each analysis.

Table 1
Physico-chemical characteristics of two Spanish prickly pears (*Opuntia ficus-indica*).

Physicochemical parameter[a,b]	*Sanguinos* (red) Variety	*Verdal* (orange) Variety
Total weight (g)	112.94 ± 16.10a	100.51 ± 13.11a
Apical caliber (cm)	7.28 ± 0.80a	7.19 ± 0.70a
Equatorial caliber (cm)	4.72 ± 0.40a	4.55 ± 0.46a
pH	5.35 ± 0.12a	5.89 ± 0.08b
Soluble solids (°Brix)	11.20 ± 0.10a	12.40 ± 0.20b

[a] Values are the mean of at least three independent determinations ± standard deviation.
[b] Lowercase letters indicate statistically significant differences ($p < 0.05$) between varieties.

2.2. Reagents

Tetrahydrofuran (THF), methyl *tert*-butyl ether (MTBE), methanol (MeOH) and diethyl ether were purchased from VWR International (Radnor, Pensilvania, USA); anhydrous sodium sulfate, potassium hydroxide (KOH) and sodium chloride (NaCl) from Panreac Química (Barcelona, Spain); butylated hydroxytoluene (BHT) and magnesium carbonate from Acros Organics (New Jersey, USA). Standards for lycopene (L9879, ≥90%, from tomato), lutein (X6250 from marigold) and *trans*-β-apo-8′-carotenal (10810,≥96%, (UV)) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA); (all-E-)-β-carotene (HPLC 96%, synth., cryst.), (all-E-)-α-carotene (HPLC 97%, synth., cryst.), (all-E-)-β-cryptoxanthin (HPLC 97%, synth., cryst.), (all-E-)-zeaxanthin (HPLC 97%, synth., cryst.), (all-E-)-neoxanthin (HPLC 97%, isolated, cryst.) and (all-E-)-violaxanthin (HPLC 95%, isolated, cryst.) from CaroteNature (Ostermundigen, Switzerland).

2.3. Carotenoid extraction and saponification

Extraction and saponification procedures were carried out under dim light. Freeze dried samples were turned into a fine powder using a cutter mill. 1 g of freeze-dried sample was combined with 0.5 g of magnesium carbonate and 60 μL of trans-β-apo-8′-carotenal (0,2 mg/mL), as internal standard, and then extracted with 20 ml of THF stabilized 0.01% BHT in a homogenizer (OMNI Macro ES®, OMNI International). The extract was then filtered and the residue was washed with another 20 mL of THF and filtered again. Both filtrates were combined and evaporated to half the volume on a rotatory evaporator at 35 °C. The concentrated extract was then added to a funnel containing 15 mL of diethyl ether and 25 mL of water saturated with NaCl. The organic phase was separated and dried with anhydrous sodium sulfate. The dried organic phase (non-saponified extract) was completely evaporated by vacuum and controlled temperature (20 °C) and then, dissolved to exactly 2 mL with MeOH/MTBE/H_2O (45.5:52.5:2, v/v/v), filtered through a 0.45 μm membrane filter and immediately analyzed by HPLC.

In the case of saponified extracts, the dried organic phase was combined with 4 mL of 30% methanolic potassium hydroxide and kept under magnetic agitation for 1.5 h in nitrogen atmosphere in the dark. The saponified extract was added to a funnel containing 15 mL of diethyl ether and was washed five times with 25 mL of water saturated with NaCl, discarding the aqueous phase each time, to obtain a neutral pH. The extract was then dried with anhydrous sodium sulfate, completely evaporated on a rotatory evaporator with controlled temperature (20 °C) and then, re-dissolved to 2 mL with MeOH/MTBE/H_2O (45.5:52.5:2, v/v/v), filtered through a 0.45 μm membrane filter and immediately analyzed by HPLC.

2.4. Carotenoid analysis by HPLC-PDA

The identification and quantification of carotenoids in saponified and non-saponified prickly pear extracts was carried out using a 1200 Series Agilent HPLC System (Agilent Technologies, Santa Clara, CA, U.S.A) with a reverse phase C30 column (YMC-Pack YMC C30, 250 × 4.6 mm i.d., S-5 μm, YMC Co., Ltd). The solvents used for separation consisted on a mix of Methanol/MTBE/Water (81:14:4, v/v/v, eluent A) and Methanol/MTBE (10:90, v/v, eluent B) both containing 0.1% of ammonium acetate. The elution gradient was linear, starting at 100% A and ending with 100% B, in 60 min. Flow rate was 1 mL/min and the column temperature was 32 °C. Injection volume was 20 μL. Carotenoids were monitored at 450 nm; additional UV/Vis spectra was recorded between 300 to 700 nm.

The individual carotenoid identification was carried out by comparing retention times, UV–Vis absorption and mass spectra to those authentic standards. When reference compounds were unavailable, obtained UV/Vis absorption and mass spectra were compared to those reported in literature (De Faria, De Rosso, & Mercadante, 2009). Prior to quantification by HPLC-PDA, the concentrations of stock solutions of lycopene, lutein, *trans*-β-apo-8′-carotenal, (all-E-)-β-carotene, (all-E-)-α-carotene, (all-E-)-β-cryptoxanthin, (all-E-)-zeaxanthin, (all-E-)-neoxanthin and (all-E-)-violaxanthin were determined spectrophotometrically using their specific absorption coefficients according to Britton (1995) in order to elaborate linear calibration curves. These calibration curves (up to seven concentration levels) were prepared with standard stock solutions for each carotenoid in the concentration range 5–100 μg/mL. Calibration curves were constructed by plotting the peak area at 450 nm for all carotenoids.

The (all-E-)-β-carotene calibration was used for quantitation of β-carotene, β-carotene-isomers, while (all-E-)-violaxanthin, violaxanthin-isomers and (all-E-)-antheraxanthin were quantitated by violaxanthin calibration. In addition, (all-E-)-lutein calibration was used for lutein-epoxide quantitation. Other carotenoids as (all-E-)-neoxanthin, (all-E-)-β-criptoxanthin and lycopene was quantitated by the corresponding standards. Results were expressed micrograms of the corresponding the carotenoid per 100 g of fresh weight. Chlorophylls compounds are not quantified in the *Opuntia* peel extracts.

2.5. Liquid chromatography-mass spectrometry (LC-MS (APCI$^+$))

LC/MS analyses were performed with the same HPLC system described above coupled on-line to an Agilent mass spectrometry detector with APCI source model G1947B compatible with the LCMS SQ 6120 equipment, according to the procedure described by Breithaupt and Schwack (2000). Positive ion mass spectra of the column eluate of 13,000 Th/s (peak width 0.6 Th, FWHM). Nitrogen was used both as the drying gas at a flow rate of 3.5 L/min and as nebulizing gas at a pressure of 50 psi. The nebulizer temperature was set at 350 °C and a potential of +2779/−2779 kV was used on the capillary. Corona was set at 2000 nA both in positive and negative ion mode, and the vaporizer temperature was set at 400 °C. Collisium gas was helium and fragmentation amplitude was 0.8–1.2 V. The chromatographic conditions were the same as described for quantitative analyses of carotenoids. The HPLC retention times, UV/Vis spectra, and MS spectral data of carotenoids from whole fruit, peel and pulp of two varieties of Spanish prickly pears (*Opuntia ficus-indica*) are showed in Table 2.

2.6. Vitamin C analyses

Vitamin C was analyzed by high performance liquid chromatography according to the method proposed by Sánchez-Moreno, Plaza, De Ancos, and Cano (2006) with light modifications. 1 g of freeze-dried sample was extracted with a 3% metaphosphoric acid and 8% acetic acid solution. The sample was centrifuged during 10 min at 10,000 rpm at 5 °C (Hettich Zentrifugen MIKRO 200R®). The supernatant was separated and diluted to 10 mL volume with distilled water. A Zorbax SB-C-184.6 × 250 nm y 5 μm column was used. The mobile phase was 20 mM KH_2PO_3 solution at 2.5 pH with phosphoric acid and methanol (80:20), which was eluted during 15 min at room temperature. The flow rate was 1 mL/min and the injection volume was 20 μL. A UV–Vis detector was used and the absorbance was determined at 245 nm.

2.7. Total phenolics analyses

Phenolic compounds were analyzed spectrophotometrically with the Folin-Ciocalteu reagent according to the procedure pro-

Table 2
HPLC retention times, UV/Vis spectra, and MS spectral data of carotenoids from Spanish prickly pears (*Opuntia ficus-indica*).

Peak[a]	Rt (min)	Compound Identity	λ_{max} (nm)	λ_{max} (nm) according to bibliography in acetone[b]	% III/II	Epoxide test	HPLC/APCI(+) MS fragmentation pattern (*m/z*)	
							$[M + H]^+$	Characteristic fragments
1	6.26	(all-E)-violaxanthin	412, 436, 464	421,442,473	98	+	601	583 $[M + H\text{-}H_2O]^+$, 565 $[M + H\text{-}2H_2O]^+$,
2	6.52	(all-E)-neoxanthin	412, 434, 464	418,442,471[c]	99	+	601	583 $[M + H\text{-}H_2O]^+$, 565 $[M + H\text{-}2H_2O]^+$, 547 $[M + H\text{-}3H_2O]^+$
3	7.17	(9Z)-violaxanthin	(330), 411, 432, 462	411,435,465	89	+	601	583 $[M + H\text{-}H_2O]^+$, 565 $[M + H\text{-}2H_2O]^+$
4	8.33	(all-E)-anteraxanthin	419, 444, 470	422,444,474[c]	66	+	585	567 $[M + H\text{-}H_2O]^+$, 549 $[M + H\text{-}2H_2O]^+$,
5	9.05	(9Z)-neoxanthin	(328),416,440, 464	418,439, 468	55	+	601	583 $[M + H\text{-}H_2O]^+$, 565 $[M + H\text{-}2H_2O]^+$, 547 $[M + H\text{-}3H_2O]^+$
6	10.03	(all-E)-lutein	423, 444, 472	424,445,474[c]	62	–	569	551 $[M + H\text{-}H_2O]^+$, 533 $[M + H\text{-}2H_2O]^+$
I*	10.41	Chlorophyll b	342, 464, 649	430,453,642[d]		–	909	631 $[M + H\text{-}C_{20}H_{38}]^+$, 613 $[631\text{-}H_2O]^+$
7	11.26	(all-E)-zeaxanthin	426, 450,474	430,452,479	18	–	569	551 $[M + H\text{-}H_2O]^+$, 533 $[M + H\text{-}2H_2O]^+$
8	12.33	Lutein-5,6-epoxide	418, 442, 468	418,441,471	85	+	585	567 $[M + H\text{-}H_2O]^+$, 549 $[M + H\text{-}2H_2O]^+$
9	13.81	Apocarotenal (internal standard)	462	465		–	417	399 $[M + H\text{-}H_2O]^+$, 325 $[M + H\text{-}74]^+$
10	17.88	(all-E)-β-criptoxanthin	428, 450, 477	428,450,478	18	–	553	535 $[M + H\text{-}H_2O]^+$, 461 $[M + H\text{-}92]^+$
11	22.41	(all-E)-α-carotene	337, 410, 445, 468	335, 422, 442, 471[c]	61	–	537	457 $[M + H\text{-}80]^+$, 413 $[M + H\text{-}124]^+$, 177 $[M + H\text{-}360]^+$, 137 $[M + H\text{-}400]^+$, 123 $[M + H\text{-}414]^+$
II*	23.09	Chlorophyll a	368, 408, 665	410, 430, 660[d]		–	893	615 $[M + H\text{-}C_{20}H_{38}]^+$
III*	23.38	Chlorophyll a′	364, 408, 665 408, 665	410,426, 660[d]		–	893	615 $[M + H\text{-}C_{20}H_{38}]^+$
12	25.60	(all-E)-β-carotene	428, 450, 476	429,452,478	16	–	537	457 $[M + H\text{-}80]^+$, 445 $[M + H\text{-}92]^+$, 400 $[M + H\text{-}137]^+$, 269 $[M + H\text{-}268]^+$, 177 $[M + H\text{-}360]^+$, 137 $[M + H\text{-}400]^+$
13	26.90	(9Z)-β-carotene	(335), 423, 444, 472	(334), 424,448,474	58	–	537	457 $[M + H\text{-}80]^+$, 445 $[M + H\text{-}92]^+$, 400 $[M + H\text{-}137]^+$, 269 $[M + H\text{-}268]^+$, 177 $[M + H\text{-}360]^+$, 137 $[M + H\text{-}400]^+$
14	44.27	Lycopene	446,472, 502	447,472,504	6	–	537	457 $[M + H\text{-}80]^+$, 413 $[M + H\text{-}124]^+$, 177 $[M + H\text{-}360]^+$, 137 $[M + H\text{-}400]^+$, 121 $[M + H\text{-}416]^+$

* Chlorophylls compounds.
[a] Peak numbers are according to Fig. 1.
[b] Britton et al. (2004).
[c] Measured in ethanol.
[d] Measured in diethyl ether.

posed by Stintzing et al. (2005). 0.7 g of freeze-dried sample was extracted with 10 ml of methanol and homogenized at 7500 rpm for 4.5 min and, later centrifuged at 12,000*g* during 15 min. The supernatant was diluted to 10 mL volume with methanol. The reactants were placed in a test tube in the following order: 150 μL sample, 750 μL Folin reagent (previously diluted 10 times in distilled water) and 600 μL of 7.5% saturated sodic carbonate solution. The samples were stirred and stored 30 min in darkness. The spectrophotometric determination was performed in a 96-microwell plate by placing 300 μL of sample and a reagent blank in corresponding microwells. The reading was done at 756 nm spectrophotometrically (Varioskan Flash de Thermo Electron Corporation®) and analyzed with SkanIt Re for Varioskan 2.4.1® software. The extraction and reaction was done by duplicate and the spectrophotometric reading of each was done by triplicate. Total phenolics were expressed as mg gallic acid per 100*g* of fresh weight.

2.8. Betalain analyses

The betalain determination was done spectrophotometrically by an adaptation of the method proposed by Cai, Sun, and Corke (1998). 1 g of freeze-dried extract was added to 10 mL of distilled water and homogenized at 7500 rpm during 4.5 min and centrifuged at 12,000*g* during 15 min. The supernatant was separated and diluted to a 10 ml volume. The samples were analyzed in a cell spectrophotometer (SmartSpec Plus BIO-RAD®) at 535 nm for betacyanin detection and at 483 nm for betaxanthin detection. The following equation was employed for sample quantification.

$$BC(mg/g) = [A(Df)(Mw)(Vd)/(\varepsilon(L)(Wd)$$

where A represents the absorption at the respective wavelength determination, Df is the dilution factor, Mw the molecular weight of betanin (550,000 mg/mol) or indicaxanthin (308,000 mg/mol), Vd the dilution volume, ε is the molar extinction coefficient (betanin λ = 538 nm; 60,000 L/mol cm in water) and indicaxanthin (λ = 480 nm; 48,000 L/mol cm in water). L is the light width pathlength and Wd the sample weight. The results were expressed in betanin mili-equivalents for betacyanin content and in indicaxanthin mili-equivalents for betaxantin content.

2.9. Antioxidant activity

The 2,2-difenil-1-picrylhydrazyl radical (DPPH**.**) assay was performed according to the method described by Vázquez-Gutiérrez et al. (2013). 0.7 g of freeze-dried sample was added to 10 mL of methanol and homogenized at 7500 rpm for 4.5 min and centrifuged at 12,000*g* during 15 min. The supernant was diluted to 10 mL volume with methanol. 40 μL of sample were added to 1160 μL of 10 μM 2,2-difenil-1-picrylhydrazyl solution (DPPH·) and incubated during 60 min in darkness. Afterwards 300 μL were placed in a 96 microwell plate and absorbance was determined spectrophotometrically at 515 nm.

The oxygen radical antioxidant capacity (ORAC) was determined with the method proposed by Ou, Huang, Hampsch-

Woodill, Flanagan, and Deemer (2002) in a microwell plate. 1 g of freeze-dried sample was added to 10 mL of distilled water and homogenized at 7500 rpm during 4.5 min and centrifuged at 12,000*g* during 15 min. The supernatant was separated and diluted to a 10 mL volume. In this analysis a PBS 0.075 M buffer at pH 7.4 with potassium phosphate (KH_2PO_4) and sodium phosphate (NaH_2PO_4) was used. Samples were diluted accordingly and the Trolox curve was prepared using concentrations from 2 to 10 mM. 20 μL of diluted sample or Trolox were placed in each microwell, 120 μL of a 4 × 10-3 μM fluorescein solution was added. The micro plate was incubated at 37 °C during 10 min. Afterwards, 60 μL of a 153 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) solution were added and the micro plate was read at a 485 nm excitation wavelength and a 530 nm emission wavelength once every minute during 47 min at 37 °C. The data was analyzed by adding the area under the curve (AUC) in a fluorescence vs time graph and subtracting the blank.

2.10. Statistical analysis

The compositional data were expressed as mean and standard deviation (SD). The obtained results were evaluated with variance analysis (ANOVA) and the least significant differences (LSD) were calculated at a $p < 0.05$ significance level. The correlation coefficients were determined by Pearson's test at a $p < 0.05$ significance level. The statistic software employed was SPSS version 2.7. All the analysis was done at least in triplicate.

3. Results and discussion

3.1. Characterization of the carotenoid profile of Spanish prickly pear fruits (Opuntia ficus-indica, spp.)

The carotenoid pigments in the Spanish most abundant prickly pear fruits, red and orange coloured flesh (see Figs. 3 and 4 in the Supplementary material for illustration of the fruit color), were characterized and identified according to their chemical, chromatographic and spectroscopic properties (UV–vis and mass spectroscopic analysis). Figs. 1 and 2 show the reversed C_{30} HPLC chromatograms corresponding to the direct (A) and the saponified (B) carotenoid extracts obtained from peels of mature prickly pear fruits of both varieties. After saponification only the disappearance of chlorophyll compounds were observed. In the pulp or flesh of the Opuntia fruits extracts similar results were obtained. In all direct extracts analyzed by HPLC method employed in this work, any xantophyll esters were detected.

The two classes of carotenoids are carotenes and xanthophylls, which are oxygenated derivatives. In fruits and vegetables xanthophylls are present either in a free, unesterified form, or as esters with fatty acids. In some foods like corn, spinach, broccoli or other green leafy vegetables, xanthophylls are present exclusively in unesterified form. In other fruits and vegetables such as pepper, wolfberry, sea buckthorn, apple, squash etc., xanthophylls are mostly found in esterified form (Pérez-Galvez & Minguez-Mosquera, 2002).

Few published papers described the composition of carotenoids of prickly pear fruits (Yahia et al., 2010) or *Opuntia* cladodos derivatives as marmalades (González-Cruz, Filardo-Kerstupp, Bello-Pérez, Güemes-Vera, & Beranrdino-Nicanor 2012). However, in the study conducted by Yahia et al. (2010), the saponification of the *Opuntia* extracts were not made, and only the composition by direct analysis of carotenoids in *Opuntia* pulp (or flesh) tissue is described. In all Mexican cultivars studied in this work, the carotenoid pattern was dominated by lutein and neoxanthin as the principal carotenoid pigments, and β-carotene was not detected in this study in any Mexican cultivar or lines of prickly pear fruit pulps. Also, chlorophyll a was described as the principal pigment of chlorophyll group in all cultivars/lines, found in the HPLC-ESI-MS analysis of extracts some peaks corresponding to chlorophyll a and pheophytin a and b, these lasts in trace amounts.

In the study about *Opuntia* cladodos (nopal) low-calorie marmalade (González-Cruz et al., 2012), the HPLC method employed used a C_{18} column, being lutein, β-carotene and β-criptoxanthin the most abundant carotenoids in fresh nopal and marmalades. In this work, only these three carotenoids were identified and quantified.

The profile of carotenoids in Spanish varieties of prickly pear includes 9 xanthophylls and 4 carotenes (lycopene) (Table 2). On one hand, the xanthophylls represent 93.6 and 92.7% of the total carotenoids present in the whole fruit of red and orange *O. ficus-indica* prickly pears, respectively. Whereas (all-E-)-lutein is present in high quantities and represents 71 and 72% of the total saponified carotenoid content, in red and orange prickly pears (whole fruit), respectively. Besides (all-E)-lutein, the dominant xanthophylls in both varieties were (all-E)-violaxanthin and (all-E-)-zeaxanthin. Mondragon-Jacobo et al. (2009) analyzed 10 cultivars and lines of Mexican prickly pear with the fruit of purple, red, orange, yellow and white pulp colors where they identified only 5 xantophylls. The study detected (all-E)-lutein as the principal carotenoid in all 10 cultivars and reported it as present in moderate amount. The other carotenoids identified by these authors were neoxanthin, violaxanthin (in their *-trans* and *-cis* states) as well as lutein. In the present study (Table 2), the hydrocarbon carotenes were present in smaller quantities compared to the xanthophylls. The identified carotene species were (all-E)-α-carotene, (all-E)-β-carotene and (9Z)-β-carotene and lycopene. Xantohphylls, under reversed-phase conditions of HPLC analysis have shorter retention times than hydrocarbon carotenes, being lycopene the most retained compound (Fig. 1 and 2).

The identification of individual free carotenoids was carried out in direct and saponified extracts obtained from peel, pulp and whole prickly pear fruits, and also chlorophylls were identified in direct extracts (non saponified), in order to determine near to complete native profile of these pigments in Spanish Opuntia fruit tissues. Table 2 shows the identification for each peak of a total of 14 carotenoid compounds (including *trans*-β-apo-8′-carotenal as internal standard) and 3 chlorophyll compounds, indicating their retention time in the C*30* reverse-phase column, Uv–vis absorption, and%III/II index, and the characteristic fragments in APCI(+) mass spectrometry. The use of authentic carotenoid standards as lycopene, (all-E)-lutein, (all-E)-β-carotene, (all-E)-α-carotene, (all-E)-β-cryptoxanthin, (all-E)-zeaxanthin, (all-E)-neoxanthin and (all-E)-violaxanthin, and *trans*-β-apo-8′-carotenal as internal standard make easy the identification and quantification of these carotenoids in the extracts.

Peaks **1** (Rt = 6.26 min) and peak **3** (Rt = 7.17 min) were identified as (all-E)- violaxanthin and (9Z)-violaxanthin, respectively. The UV–visible spectrum of peak **1**, with λ_{max} at 412, 436, 464 nm, and the characteristic pronounced fine structure (%III/II = 98) was in agreement with the chromophore consisted of nine conjugated double bonds and at least one β-ring, comparison with the authentic standard and data reported in the literature (Britton, 1995). In contrast, peak **3** showed an UV–visible spectrum with a less marked fine structure and the appearance of a "cis peak" at 330 nm (330, 411, 432, 462 nm;%III/II = 89). Both peaks disappeared upon treatment of the extract with diluted HCl (epoxide test). Similar process carried out in peak **2** (Rt = 6.52 nm) identified as (all-E)-neoxanthin (λ_{max} at 412, 434, 464) and peak **5** (Rt = 9.05 min) identified as (9Z)-neoxanthin (λ_{max} at 328, 416, 440, 464), with the subsequent formation of a new peak, corre-

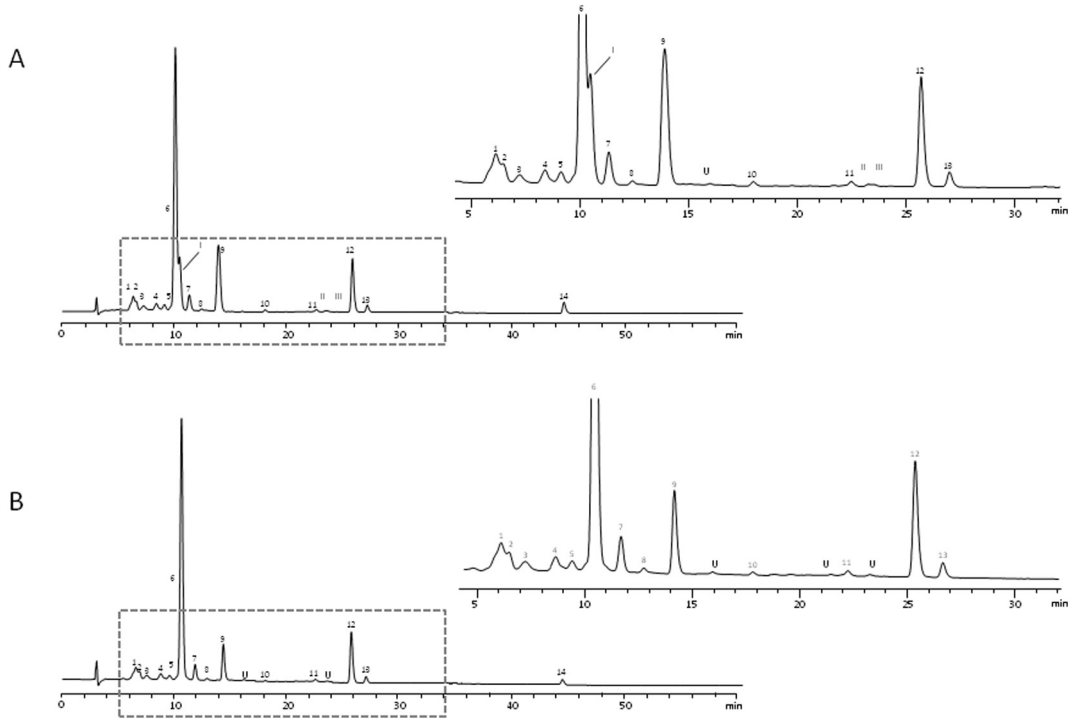

Fig. 1. C30 reversed-phase HPLC chromatogram obtained from direct (A) and saponified (B) carotenoid extracts from Spanish prickly pear (*Opuntia ficus-indica*) peel, *Sanguinos* (red) variety. UV–vis detection at 450 nm. Peak identities in Table 2. (U) un-identified compound.

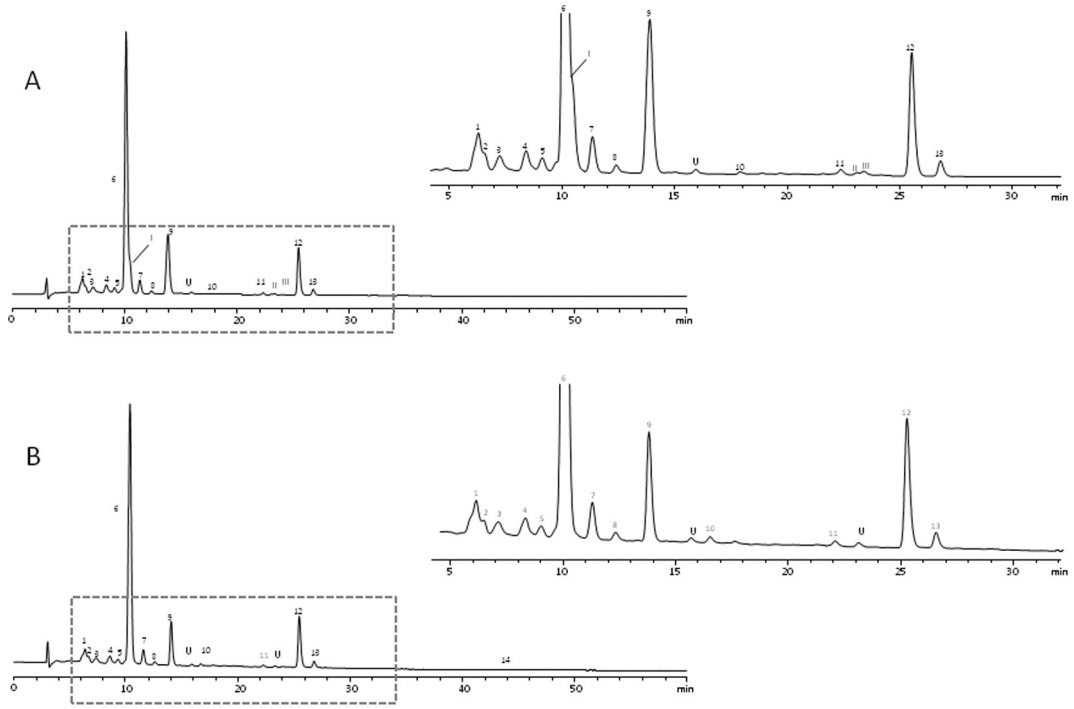

Fig. 2. C30 reversed-phase HPLC chromatogram obtained from direct (A) and saponified (B) carotenoid extracts from Spanish prickly pear (*Opuntia ficus-indica*) peel, *Verdal* (orange) variety. UV–vis detection at 450 nm. Peak identities in Table 2. (U) un-identified compound.

sponding to a neochrome, generated by acid-catalysed rearrangement of one 5,6-epoxy group. Neochrome was not detected in native any prickly pear extract, nor peel, pulp or whole fruit. The LC/MS (APCI+) spectra of peaks **1** and **3** showed a protonated molecule $[M + H]+$ at m/z 601, with was consistent with the molecular formula $C_{40}H_{56}O_4$ (M_w = 601.4257), and with the authentic standard that showed also these $[M + H]^+$ value. Also the presence of various less abundant fragments at 583 $[M + H\text{-}H2O]^+$ and 565 $[M + H\text{-}2H2O]^+$, derived from neutral losses of water molecules, confirmed the presence of two hydroxyl groups. Similar characteristics showed the carotenoid compound of peak **2**, identified as (all-E)-neoxanthin, with three fragments corresponding to the

presence in the molecule of three hydroxyl groups, 583 [M + H-H2O]$^+$, 565 [M + H-2H2O]$^+$, and 547 [M + H-3H2O]$^+$.

Peak **4** (Rt = 8.33 min) was identified as (all-E)-antheraxanthin. The Uv–visible spectrum, with λ_{max} at 419, 444, 470 nm (%III/II = 66). The MS (APCI+) spectrum of antheraxanthin showed a major ion for the protonated molecule [M + H]+ at 585, which was consistent with the molecular formula C40H56O3 (Mw = 584.4229). Also, other fragments were 567 [M + H-H2O]+ and 549 [M + H-2H2O]+, related to the loss of water molecules due to the presence of two hydroxyl groups located in β-ring. Some published studies using MS (APCI+) reported that cis-antheraxanthin did not give these two fragments possible due to the low stability of these ions under the specific ionization conditions used (Meléndez-Martínez, Britton, Vicario, & Heredia, 2005). In the present study, cis- antheraxanthin did not be identified in any prickly pear fruit tissues.

Peak **6** (Rt = 10.03 min), was identified as (all-E)-lutein, showing a Uv–visible spectrum, with λ_{max} at 423, 444, 472 nm, by comparison by co-chromatography with a commercial pure standard lutein sample. Also, this compound exhibited a marked spectroscopic fine structure (%III/II = 62), which was consistent with a chromophore with nine conjugated double bonds and a potential one β-ring and one ε-ring (Britton, 1995). Several authors shown in their works (Delgado-Pelayo, Gallardo-Guerrero, & Hornero-Méndez, 2016), the APCI(+) mass spectrum of lutein can be used for unmistakable determination of the chemical structure of lutein. The main characteristic was the presence of a protonated molecule [M + H]$^+$ at *m/z* 569 and a most abundant fragment appeared at *m/z* 551, with was produced by the loss of one water molecule 551 [M + H-H2O]$^+$, followed by a fragment at *m/z* 533 [M + H-2H2O]$^+$, a loss of two water molecules with very low intensity.

Peak **7** (Rt = 11.26 min) showed a chemical, chromatographic and spectrophotometric properties that was correlated to identify as (all-E)-zeaxanthin. Uv–visible spectrum with λ_{max} at 426, 450, 474 nm, and a fine structure (%III/II = 18), that could indicate the presence of two β-ring and nine conjugated double bonds (Britton, 1995). Also, this carotenoid chemical structure was compared by co-chromatography with a commercial pure standard sample of (all-E)-zeaxanthin. Protonated molecule [M + H]$^+$ of MS spectrum, showed a value of *m/z* 569, which is consistent with the molecular formula $C_{40}H_{56}O_2$ (Mw = 582.4229). The most important MS fragments were *m/z* 551 [M + H-H2O]$^+$ and *m/z* 533 [M + H-2H2O]$^+$ related to the losses of one or two water molecules, confirming the existence of two hydroxyl groups in the chemical structure of this carotenoid.

Peak **8** (Rt = 12.33 min) was identified as lutein-5,6-epoxide, by its spectroscopic characteristics, λ_{max} at 418, 442, 468 nm (%III/II = 85) and MS spectrum, showing a major ion for the protonated molecule [M + H]$^+$ at *m/z* 585 and ion fragments, 567 [M + H-H2O]$^+$, 549 [M + H-2H2O]$^+$, 505 [M + H80]$^+$. Peak **9** (Rt = 13.81 min) was *trans*-β-apo-8′-carotenal, added to the extracts to check the recovery of carotenoids in the extraction process. Table 2 shows the spectrophotometric characteristics of this carotenoid.

The structural assignment of (all-E-)-β-criptoxanthin for peak **10** (Rt = 17.88 min) was based on the HPLC co-elution with an authentic commercial standard. The UV–visible spectrum showed λ_{max} at 428, 450, 477 nm (%III/II = 18), that indicated a chromophore consisted of nine double bonds and two β-rings. Mass spectrum was characterized by a protonated molecule [M + H]$^+$ at *m/z* 553, corresponding to a molecule $C_{40}H_{56}O$ and a fragment of *m/z* 535 [M + H-H2O]$^+$ (loss of a water molecule) indicating the presence of an only hydroxyl group, and *m/z* 461 [M + H-92]$^+$, corresponding to the loss of toluene (Breemen, Dong, & Pajkovic, 2012).

Peak **11** (Rt = 22.41 min) was identified as (all-E)-α-carotene by comparison in the first step with an authentic commercial standard of this carotenoid. The UV–visible spectrum showed λ_{max} 337, 410, 445, 468 nm (%III/II = 61), showing the appearance of a "cis peak" at 337 nm. Most of the fragment ions observed in the MS APCI (+) mass spectrum of (all-E-)-α-carotene were the same as those for (all-E-)-β-carotene (e.g., *m/z* 137, *m/z* 413 and *m/z* 457). However, the most abundant fragment ion in the positive ion tandem mass spectrum of (all-E)-α-carotene, corresponding to the α-ionone moiety of *m/z* 123, was not observed in the APCI (+) mass spectrum of (all-E-)-β-carotene. Formation of the ion of *m/z* 123 was facilitated by the position of the double bond in the terminal ring, which helped stabilize the resulting carbocation. Since this ion was not observed in the positive ion APCI tandem mass spectrum of β-carotene, γ-carotene, or lycopene, it may be used to distinguish α-carotene from these isomeric carotenes (Breemen et al., 2012).

Peak **12** (Rt = 25.60 min) corresponded to (all-E-)-β-carotene showing an UV–visible spectrum characteristics of λ_{max} 428, 450, 476 min (%III/II = 16), and identified also by HPLC co-elution with an authentic commercial standard. The mass spectrum showed the expected protonated molecule [M + H]$^+$ at *m/z* 537, with a typical fragment corresponding to the loss of toluene *m/z* 445 [M + H-92]$^+$.

Also, a hydrocarbon carotenoid was identified in peak **13** (Rt = 26.90 min), a isomer of β-carotene, which was (9Z)-β-carotene. Reverse-phase column facilitate the efficient separation of carotene isomers (Fig. 1 and 2). The UV–visible spectrum showed λ_{max} 335, 423, 444, 472 nm (%III/II = 58), showing the appearance of a "cis peak" at 335 nm. As the observed MS fragments of (all-E-)-β-carotene, the MS spectrum of (9Z)-β-carotene showed similar ions, being the most characteristic but with low abundance, *m/z* 445 [M + H-92]$^+$ by elimination of neutral molecule of toluene, and *m/z* 457 [M + H-80]$^+$ by loss of methylcyclopentadiene.

Finally, peak **14** (Rt = 44.27 min) was identified in first step as lycopene by comparison by co-chromatography with a commercial pure commercial standard lycopene. The UV–visible spectrum showed λ_{max} 446,472, 502 nm (%III/II = 6). The MS spectrum of lycopene was represented by *m/z* 457 [M + H-80]$^+$, 413 [M + H-124]$^+$, 177 [M + H-360]$^+$, 137 [M + H-400]$^+$, 121 [M + H-416]$^+$, being the fragment *m/z* 121 [M + H-416]$^+$. Lycopene fragmented during positive ion APCI(+) mass spectrometry to form numerous low mass ions (*m/z* 121, *m/z* 137 and *m/z* 177) that represented cleavages of the polyene chain. Loss of methylcyclopentadiene was observed at *m/z* 457 but not loss of toluene at *m/z* 445 (this fragment was present in the MS spectrum of β- and α-carotene (Breemen et al., 2012).

Also, in the extracts of prickly pear fruit tissues, mainly in peel, several **chlorophyll compounds (peaks I, II and III)** were identified (Fig. 1 and 2). Peak **I** (Rt = 10.41 min) showed λ_{max} 342, 464, 649 nm and was identified as chlorophyll b by comparison with data reported in the literature (Lichtenthaler & Buschmann, 2001). The MS spectrum of of chlorophyll b showed a protonated molecule [M + H]+ at *m/z* 909 which was consistent with the molecule of $C_{55}H_{70}MgN_4O_6$, showing MS APCI (+) fragments 631 [M + H-$C_{20}H_{38}$]$^+$, 613 [631-H_2O]$^+$. Peak **II** (Rt = 23.09 min) showed λ_{max} 368, 408, 665 nm and was identified by reported data and MS APCI (+) a protonated molecule at *m/z* 893 and a principal fragment 615 [M + H-$C_{20}H_{38}$]$^+$ as chlorophyll a ($C_{55}H_{72}MgN_4O_5$). In addition, peak **III** (Rt = 23.38 min) showed λ_{max} 364, 408, 665 nm and identical MS APCI (+) spectrum than chlorophyll a, which in accordance with literature data. Yahia et al. (2010) reported the presence of chlorophyll a and pheophytins a and b in the HPLC analysis of the extracts of Mexican prickly pear fruit pulp. In any Spanish prickly pear fruit tissues, no peel nor pulp, pheophytins were detected, possibly due to the process of extraction made in controlled conditions (nitrogen atmosphere, low room temperature and darkness). Supple-

mentary material Fig. 5 showed the differences among UV–vis spectrum of (all-E-)-lutein (peak **6**) and chlorophyll b (peak **I**) and Fig. 6 showed UV–vis spectrum of chlorophyll a (peak **II**) and chlorophyll a′ (peak **III**).

Saponification of the prickly pear fruit extracts produced the loss of chlorophyll compounds. Fig. 1 show the chromatograms of peel extracts of direct (A) and saponified (B), of *Sanguinas* (red) and *Verdal* (orange) prickly pear cultivars. Peaks I, II, and III identified as chlorophyll b, chlorophyll a and chlorophyll a′ disappeared after the saponification. Chlorophyll can be saponified in the presence of sodium hydroxide, leading to the production of water-soluble chlorophyllin and phytol ($C_{55}H_{72}O_5N_4Mg + 2NaOH = C_{34}H_{30}O_5N_4MgNa_2 + 2CH_3OH + C_{20}H_{39}OH$). Based on the saponification reaction, chlorophyll can be easily separated from other liposoluble biochemical compounds as carotenoids. Accordingly, this saponification treatment is often used in the determination of carotenoids content (Rodriguez-Amaya, 2001).

3.2. Carotenoid composition in Spanish prickly pear fruits (Opuntia ficus-indica, spp.)

The quantitation of carotenoids in the mature Spanish prickly pear fruit tissues are showed in Table 3. Total carotenoid content was greater in peel extracts of both prickly fruit cultivars, being 478.11 ± 3.01 and 444.90 ± 2.89 μg/100 g fresh weight, for *Sanguinos* (red) and *Verdal* (orange) prickly pear fruits respectively. This higher concentration of carotenoids in the peel comes as no surprise since carotenoids play an important role in attracting animals so they can act as pollinators and seed dispersion vehicles (Britton, Liaaen-Jensen, & Pfander, 2004). Total carotenoid content in fruit pulp ranged 255.93 ± 2.89 (*Sanguinos* fruits) and 379.45 ± 4.07 μg/100 g (*Verdal* fruits) fresh weight, showing that Orange fruits are 48% richest in these bioactive compounds. All extracts from peel and pulp showed that (all-E-)-lutein, (all-E-)-β-carotene and (all-E-)-violaxanthin as the major carotenoids (1132.51 ± 1.97, 200.4 ± 2.83 and 93.64 ± 1.87 μg/100 g fresh weight for peel of *Sanguinos* (red) fruits; and (767.98 ± 2.20, 173.50 ± 2.30 and 87.67 ± 3.01 μg/100 g fresh weight for peel of *Verdal* (orange) fruits). These three carotenoids accounting for more than 80%, followed by (all-E-)-zeaxanthin (63.26 ± 1.13 or 52.98 ± 1.27 μg/100 g fresh weight for peels of *Sanguinos* (red) and *Verdal* (orange) cultivars), (all-E-)-anteraxanthin (39.94 ± 3.01 and 40.7 ± 1.59 μg/100 g fresh weight for peels of *Sanguinos* (red) and *Verdal* (orange) cultivars) and (all-E-)-neoxanthin (34.51 ± 1.47 and 59.05 ± 2.41 μg/100 g fresh weight for peels of *Sanguinos* (red) and *Verdal* (orange) cultivars). Other carotenoids were in low concentrations as lutein-5,6-epoxide, in peel of *Sanguinos* (red) fruits (63.26 ± 1.58 μg/100 g fresh weight) or (all-E-)-neoxanthin, for peel extracts from *Verdal* (orange) fruits (59.05 ± 2.41 μg/100 g fresh weight). Lycopene was detected only in *Sanguinos* (red) prickly pear fruits, being the peel the tissue with a significant amount 45.61 ± 2.68 μg/100 g fresh weigth, meanwhile only trace amount was detected in pulp.

As explained before, carotenoids are present in prickly pear fruits tissues in a significate lower concentration than in the corresponding peel tissues. The most abundant carotenoid in *Sanguinos* (red) prickly pear cultivar pulp extract was (all-E)-lutein (201.45 ± 2.31 μg/100 g fresh weigth) followed by (all-E)-β-carotene (37.47 ± 1.67 μg/100 g fresh weigth) and (all-E)-zeaxanthin (14.32 ± 0.83 μg/100 g fresh weigth). In contrast, in *Verdal* (orange) prickly pear pulp together with (all-E)-β-carotene (79.10 ± 2.65 μg/100 g fresh weigth) and (all-E)-violaxanthin (31.95 ± 2.76 μg/100 g fresh weigth) appeared other carotenoids in a lower concentration (9Z)-violaxanthin, (all-E)-zeaxanthin and (all-E)-neoxanthin (12.61 ± 2.03; 12.27 ± 1.09 and 12.23 ± 1.45 μg/100 g fresh weigth).

For first time, a quantification of the individual carotenoids of prickly pear fruit was made. Other authors, identified only three carotenoids (neoxantin, violaxantin and lutein) but only reported that these compounds were in trace amounts in pulp of Mexican prickly pear fruits (Yahia et al., 2010), meanwhile other studies reported the total carotenoid amount in Opuntia pulp determined by spectrophometric method (Fernández-López et al., 2010).

3.3. Other constituents of Spanish prickly pear fruits (Opuntia ficus-indica, spp.) with antioxidant activity

Most of the published works about the composition of prickly pear fruits included data about the most important constituents with antioxidant activity (Fernández-López et al., 2010) of the dif-

Table 3
Content of carotenoids[*] (μg/100 g fresh weight) ± standard deviation and retinol activity equivalents (RAE) as determined after saponification of extracts of different tissues of two varieties of Spanish prickly pear fruits (*Opuntia ficus-indica*).

Carotenoid	*Sanguinos* (red) variety			*Verdal* (orange) variety		
	Whole fruit	Pulp	Peel	Whole fruit	Pulp	Peel
(all-E)-violaxanthin	23.78 ± 2.37Aa	5.76 ± 0.91Ba	93.64 ± 1.87Ca	22.23 ± 1.54Aa	31.95 ± 2.76Bb	87.67 ± 3.01Cb
(all-E)-neoxanthin	12.24 ± 0.93Aa	2.39 ± 0.34Ba	34.51 ± 1.47Ca	7.58 ± 1.07Ab	12.23 ± 1.45Bb	59.05 ± 2.41Cb
(9Z)-violaxanthin	8.41 ± 1.65Aa	Nd.	29.55 ± 2.22Ba	10.57 ± 1.34Aa	12.61 ± 2.03A	29.49 ± 1.93Ba
(all-E)-anteraxanthin	11.83 ± 0.67Aa	5.95 ± 1.02Ba	39.94 ± 3.01Ca	10.14 ± 1.67Aa	7.64 ± 0.78Ba	40.7 ± 1.59Ca
(9Z)-neoxanthin	Tr[c]	Tr[c]	Tr[c]	Tr[c]	Tr[c]	Tr[c]
(all-E)-lutein	332.16 ± 3.87Aa	201.45 ± 2.31Ba	1132.51 ± 1.97Ca	316.36 ± 2.52Ab	203.90 ± 1.39Ba	767.98 ± 2.20Cb
(all-E)-zeaxanthin	13.50 ± 1.73Aa	14.32 ± 0.83Aa	63.26 ± 1.13Ba	14.15 ± 0.67Aa	12.27 ± 1.09Aa	52.98 ± 1.27Bb
Lutein- 5,6- epoxide	13.50 ± 2.07Aa	4.73 ± 0.46Ba	63.26 ± 1.58Ca	2.28 ± 0.65Ab	4.70 ± 0.71Ba	11.59 ± 2.05Cb
(all-E)-β-criptoxanthin	Nd.	Nd.	8.82 ± 0.62a	Nd.	Nd.	5.76 ± 0.80b
(all-E)-α-carotene	3.61 ± 0.28Aa	Nd.	10.34 ± 1.05Ba	1.99 ± 0.18Ab	5.82 ± 0.93B	8.11 ± 1.06Cb
(all-E)-β-carotene	65.81 ± 2.67Aa	37.47 ± 1.67Ba	200.4 ± 2.83Ca	53.45 ± 0.97Ab	79.10 ± 2.65Bb	173.50 ± 2.30Cb
(9Z)-β-carotene	6.75 ± 0.67Aa	Nd.	26.01 ± 1.05Ba	8.73 ± 1.42Aa	6.15 ± 1.23A	21.44 ± 2.11Bb
Lycopene	Tr[c]	Tr[c]	45.61 ± 2.68	Nd.	Nd.	Nd.
Total Carotenoids[a]	478.11 ± 3.01	255.93 ± 2.89	1693.38 ± 4.21	444.90 ± 2.89	379.45 ± 4.07	1257.74 ± 3.56
Retinol Activity[b] Equivalents[b]	6.05	3.12	19.20	4.94	7.36	16.48

Nd. Not detected.

[*] Values are the mean of three independent determinations ± standard deviation. Uppercase letters indicate statistically significant differences ($p \leq 0.05$) between tissue samples of the same fruit variety. Lowercase letters indicate statistically significant differences ($p \leq 0.05$) between varieties for the same tissue.

[a] Excluding eventually unidentified carotenoids.

[b] As calculated according to the guidelines of the US Institute of Medicine (2001).

[c] Tr: Traces.

ferent constituents that contribute to their antioxidant capacity. In order to know the antioxidant composition of the Spanish prickly fruit cultivars assayed in this study, a complete analysis of total betalains, total phenols and vitamin C was conducted. Also, antioxidant activity of extracts from *Sanguinos* (red) and *Verdal* (orange) cultivars of prickly fruit tissues was determined to characterize these fruits. Maturity of these two fruit cultivars was determined by complementary analysis of physico-chemical characteristics (Table 1). There were variations in the pH and soluble solids (°Brix) as a function of the prickly pear cultivars. Upon maturation, the pH in prickly pears rises from below 5 to values between 5.6 and 6.5, which will also depend on the cultivar (Parish & Felker, 1997). The soluble solid content of the Spanish Red and Orange cultivars were statistically different (11.20 and 12.40, respectively), and are in agreement with those reported for Moroccan prickly pear fruits, which ranged between 10.16 and 16.61% (El Gharras, 2009), and Mexican cultivars which ranged from 12 to 17% (Saenz-Hernandez, 1995).

Betalains are water soluble compounds present in a restricted number of families of plants from the *Caryophyllale* family. They are classified in two chemical families: betacyanins and betaxanthins with 540 and 480 nm absorption maxima. Betalains are powerful radical eliminatiors in chemical systems and act as efficient antioxidants in biological models. The *Sanguinos* (red) prickly pear fruit (*O. ficus-indica)* cultivar showed the higher concentration of betacyanins and betaxanthins than the *Verdal* (orange) cultivar (Table 4) with an average of 6.62 and 3.34 mg/100 g fresh fruit, depending on the variety or tissue. Betacyanins were concentrated on the fruit peels while the betaxanthins were distributed uniformly among the fruit, peel and pulp. The major concentration in total betalains was observed in the whole fruit samples, this was also noticeable in the objective color evaluation of the samples (whole fruit samples presented a more intense color than the rest).

Total phenols were determined by Folin-Ciocalteau assay, which detects electron transfer by measuring the reductive capacity of the sample and therefore may also be considered an antioxidant activity assay. The Spanish *Sanguinos* (red) and *Verdal* (orange) cultivars of prickly pear showed similar total phenolic content of 482.26 and 492.71 mg gallic acid equivalents/100 g fresh fruit, respectively (Table 4). These results are in agreement with the study performed by Jiménez-Aguilar et al. (2015) performed with two Mexican *O. ficus-indica* (Rojo San Martín and Cristal) prickly pear cultivars. The phenolic content was found to be higher in the peel than in the pulp. The reported phenolic content for the red Rojo San Martín and Cristal prickly pear varieties was 386 and 265 mg gallic acid equivalents/100 g of fresh pulp and 1534 and 1034 mg gallic acid equivalents/100 g fresh peel, respectively. In the present study, the total phenolics for Spanish *Sanguinos* and *Verdal* prickly pear cultivars (482.26 and 492.71 mg gallic acid equivalents/100 g fresh fruit, respectively) for whole fruit, being the concentration of peel tissues greater (698.37 and 630.30 mg gallic acid equivalents/100 g fresh peel, respectively) than the pulp ones. These values for Spanish prickly fruit cultivars were higher than those reported by Fernández-López et al. (2010) of 218, 204 and 164 mg gallic acid equivalents/100 g fresh fruit for the *O. ficus-indica, O. undulata and O. stricta* varieties, respectively, possibly due to the different maturity of the fruits. It is important to know that the Folin-Ciocolteau assay may present a slight overestimation of the actual phenolic content of the fruit tissues, due to the presence of other components with reductive capacity, but in general, this assay could be a useful assay which allows an adequate quantitative comparison between prickly pear varieties.

Vitamin C is an important nutrient with possesses significant antioxidant activity in biological systems. In prickly pear fruits, it is the third principal vitamin and its concentration is related to the cultivar of prickly pear fruit and to the fruit maturity. The vitamin C content of the studied *Sanguinos* (red) and *Verdal* (orange) cultivars of prickly pear fruits in this work was 71.94 and 66.44 mg/100 g fresh weight for whole fruit, respectively with no statistical differences among varieties (Table 4). Also, vitamin C content was higher in peel tissues than in pulp ones, being the *Verdal* (orange) fruit cultivar the richest in this vitamin, mainly in the fruit peel (123.76 mg/100 g fresh peel weight).

Table 4
Vitamin C content, total phenolics, betalains and antioxidant activities (DPPH[o] and ORAC) of two varieties of Spanish prickly pear (*Opuntia ficus-indica*).

Parameters[*]	*Opuntia* tissue	*Sanguinos* (red) Variety	*Verdal* (orange) Variety
Vitamin C[a]	Whole Fruit	71.94 ± 0.89 Aa	66.44 ± 5.44 Aa
	Pulp	47.19 ± 2.54 Ba	62.09 ± 6.57 Ab
	Peel	95.62 ± 8.04 Ca	123.76 ± 24.75 Ba
Total Phenolic Content[b]	Whole Fruit	482.26 ± 34.82 Aa	492.71 ± 14.24 Aa
	Pulp	457.25 ± 12.99 Aa	432.50 ± 11.88 Ba
	Peel	698.37 ± 29.26 Ba	630.30 ± 45.14 Ca
Betacyanins[c]	Whole Fruit	3.57 ± 0.02Aa	0.87 ± 0.06 Ab
	Pulp	1.98 ± 0.05 Ba	0.37 ± 0.04 Bb
	Peel	2.52 ± 0.10 Ca	1.17 ± 0.04 Cb
Betaxanthins[d]	Whole Fruit	3.04 ± 0.03 Aa	1.70 ± 0.05 Ab
	Pulp	2.61 ± 0.08 Ba	1.70 ± 0.04 Ab
	Peel	2.00 ± 0.15 Ca	1.73 ± 0.04 Aa
Total Betalains[e]	Whole Fruit	6.62 ± 0.04 Aa	3.34 ± 0.05 Ab
	Pulp	4.59 ± 0.07 Ba	2.07 ± 0.04 Bb
	Peel	4.54 ± 0.12 Ba	2.90 ± 0.06 Cb
Antioxidant Activity[f]			
DDPH	Whole Fruit	126.49 ± 8.48 ABa	133.07 ± 8.38 Aa
	Pulp	108.85 ± 7.51 Aa	122.47 ± 10.13 Aa
	Peel	141.80 ± 8.07 Ba	141.60 ± 8.22 Aa
ORAC	Whole Fruit	38.66 ± 7.28 Aa	36.09 ± 5.08 ABa
	Pulp	30.28 ± 4.70 Aa	28.53 ± 4.09 Aa
	Peel	88.31 ± 9.94 Ba	48.35 ± 8.45 Bb

[*] Values are the mean of at least three independent determinations ± standard deviation. Uppercase letters indicate statistically significant differences ($p < 0.05$) between tissues. Lowercase letters indicate statistically significant differences ($p < 0.05$) between varieties.
[a] mg ascorbic acid equivalents/100 g fresh weight.
[b] mg of gallic acid/100 g of fresh weight.
[c] mg of betanin/100 g of fresh weight.
[d] mg of indicaxanthin/100 g of fresh weigth.
[e] mg of total betalains content (betacianins + betaxanthins)/100 g of fresh weigth.
[f] μmol trolox equivalents/100g fresh weight.

3.4. Antioxidant activity of prickly pear fruits (Opuntia ficus-indica, spp.)extracts. Correlation among antioxidant activity and bioactive constituents

Antioxidants are capable of deactivating free radicals through two principal mechanisms, hydrogen atom transfer and individual electron transfer. On one hand, the DPPH· assay is classified as an electron transfer (ET) method and on the other hand the ORAC assay is classified as a hydrogen atom transfer (HAT) method. By the DPPH· assay, the observed antioxidant activity was 126.49 and 133.07 μmol trolox equivalents/100 g fresh weight for the *Sanguinos* (red) and *Verdal* (orange) cultivars of prickly pear fruit (whole fruit), respectively (Table 4). Fernández-López et al. (2010) reported similar values of 108 μmol trolox equivalents/100 fresh weight for *O. undulata* and lower values of 5.22 and 4.72 μmol trolox equivalents/100 fresh weight for *O. ficus-indica* and *O. stricta,* respectively.

On the other hand, ORAC assay depends on the degradation of fluorescein caused by 2,2′-azobis(2-amidinopropane) dihydrochloride (APPH) radical and the inhibition of the reaction by the presence of antioxidants in the extract. The observed value means of *Sanguinos* (red) and *Verdal* (orange) cultivars of prickly pear fruit cultivars was 38.66 and 36.09 mmol trolox equivalents/100 g fresh weight, respectively for whole fruit extracts. It has been reported that the antioxidant activity of prickly pear fruits are twice higher as the observed for other fruits as pears, apples, tomatoes, bananas or white grapes, and is comparable to the ORAC activity of red grapes, pink grapefruit and red oranges (Albano et al., 2015).

The correlation among the phytochemicals analyzed and the antioxidant activity of the prickly pear fruit extracts studied in the present work is showed in Table 5 (Supplementary material). The ORAC assay data showed the highest correlation with the total phenolic content (r = 0.92). Kuti (2004) reported similar correlation coefficients of 0.78, 0.88, 0.80 and 0.76 between ORAC antioxidant activity and total flavonoids for *O. ficus-indica*, *O. lindheimeri, O. streptacantha* and *O. stricta v. stricta*, respectively. Although, the relationship of total carotenoids with the ORAC assay data was of 0.86, any correlation must be discarded due to the lack of causality of carotenoids being present in the aqueous extracts analyzed. This high relationship among carotenoids and antioxidant activity measured with ORAC assay was also due to the observed lineal relationship between total phenolics and total carotenoids (r = 0.96). The highest correlation with the DPPH· assay was observed among the vitamin C content (r = 0.93), followed by total carotenoids (r = 0.92) and total phenolic compounds (r = 0.88). Kugler, Graneis, Stintzing, and Carle (2007) concluded that vitamin C contributes up to 68% of the antioxidant activity of prickly pears. Fernández-López et al. (2010) reported a high correlation (r = 0.97) between the DPPH· assay and the total phenolic contents of three Spanish Red-skinned prickly pears (*O. stricta, O. undulata* and *O. ficus-indica*). Betalains showed no statistically significant correlation with any of the employed antioxidant assays due to the nature of mechanisms involved in the antioxidant assay's (DPPH· and ORAC). However important to emphasize on the relevance of betalain's antioxidant activity in biological systems that have been reported in some published works, where betalains seemed to exhibit a high antioxidant and anti-inflammatory capabilities through *in vitro* and *in vivo* animal models studies (Clifford, Howatson, West, & Stevenson, 2015).

4. Conclusions

Spanish *Sanguinos* (red) and *Verdal* (orange) cultivars of prickly pears (*Opuntia ficus-indica)* contain an interesting carotenoid profile with the presence of only free xanthophylls and hydrocarbon carotenoids and a low amounts of some chlorophylls. (all-E-)-lutein is the most abundant compound which represents 71–72% of the total carotenoids, followed by (all-E-)-β-carotene which is present in lower quantity in all fruit tissues. In total 9 xanthophylls ((all-E)-violaxanthin, (all-E)-neoxanthin, (9Z)-violaxanthin, (all-E)-anteraxanthin, (9Z)-neoxanthin, (all-E)-lutein, (all-E)-zeaxanthin, lutein- 5,6- epoxide (all-E)-β-criptoxanthin), and 4 carotenes ((all-E)-α-carotene, (all-E)-β-carotene, (9Z)-β-carotene, and lycopene) were identified. The Spanish *Sanguinos* (red) and *Verdal* (orange) prickly pear fruit cultivars contained similar total carotenoid content but their distribution was significantly different among tissues, peel or pulp. The fruits also contain similar concentration of vitamin C and total phenolics. In contrast, they showed significant differences in their betalain profile and concentration. Prickly pear fruit peel contained the highest content of phytochemicals (carotenoids, vitamin C, betalains, and phenolic compounds) and is therefore of high value for the development of nutraceuticals and functional foods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.05.135.

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