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Application of high-performance liquid chromatography to the characterization of the betalain pigments in prickly pear fruits

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Abstract

The qualitative and quantitative betalain pigment content of two cultivars of prickly pear (*Opuntia ficus-indica*) fruits grown in southeastern Spain was evaluated. After methanolic extraction of crushed fruits, reversed-phase high-performance liquid chromatography and photodiode array detection were applied simultaneously for the separation, identification and quantification of these pigments. Two main pigments were obtained, which were identified as indicaxanthin (λ_{max} 484 nm) and betanin (λ_{max} 535 nm). Spectrophotometric evaluation of both pigments showed a yield of around 20–30 mg per 100 g of fresh pulp. When the influence of temperature (25 to 90°C) on betacyanin pigment stability was investigated, the results revealed a substantial degree of thermodegradation at temperatures higher than 70°C.

Keywords: *Opuntia ficus-indica*; Fruits; Pigments; Betacyanins; Betaxanthins

1. Introduction

There is growing interest in the development of natural additives for use in the food industry, which has been encouraged by a strong consumer demand for natural products. This trend includes considerable interest in obtaining food colorants from natural sources [1–4]. Natural colorants have become increasingly popular with consumers because synthetic colorants are frequently perceived as undesirable or harmful. Some synthetic colorants have been blamed for allergenic and intolerance reactions [2,3]. The consumer preference for naturally derived colorants is associated with the image of healthy living and a lifestyle of quality.

Opuntia ficus-indica is native to the Western Hemisphere, and was subsequently brought to Europe, Africa and the Middle East. Nowadays it is mainly found in the wild, although there are commercial plantations in Mexico, Brazil, Chile, Israel, Italy and Spain [5]. The prickly pear is the fruit of the genus *Opuntia*, which belongs to the Cactaceae family. It is a berry, consisting of a thick pericarp with a number of clefts of small prickles, reddish purple, yellow or white in color, with a luscious sweet pulp intermixed with a number of small seeds. Some cultivars of these fruits are characterized by an intense reddish purple color caused by the presence of betacyanin pigments [5–7].

Betacyanins are red pigments typically associated with plants of the order Centrospermae, in which

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they represent a taxonomic chemical constituent [8]. They are the main pigments of *Beta vulgaris*. Betacyanins are frequently associated with the yellow betaxanthins of similar biogenesis, and both classes are referred to collectively as betalains.

The use of betalain pigments as food additives dates back to the turn of the 20th century, when juice from pokeberries was added to wine to impart a more desirable red color [9]. Commercial and purified betalain preparations obtained from red beet extracts have been shown to be effective colorants of foods with compatible chemical and/or physical properties [10–14]. The intense reddish purple color of some cultivars of *Opuntia ficus-indica* suggests it may be of use as a natural food additive although, unlike the red beet pigments, this pigment has been much less investigated.

This study was designed to analyze chromatographically the pigment content in prickly pear fruits grown in the Region of Murcia (Spain) and to investigate the possibility of using this plant material as a source of natural colorants. To do so, we analyzed the occurrence and stability of the betalain pigments present in these fruits.

2. Experimental

2.1. Plant material

Prickly pear fruits (*Opuntia ficus-indica*), of reddish purple and yellow color, grown in Alhama, Region of Murcia (Spain) were used in this investigation. Mature fruit samples were harvested between July and September 1999 and taken immediately to the laboratory where they were manually peeled and subjected to the pigment extraction.

2.2. Pigment extraction

Freshly cut fruit flesh was homogenized in methanol, with a ratio mass fruit (g)/solvent (ml) of 1:5, for 1 min and the homogenate was passed through a 0.45-μm nylon filter (Lida, Kenosha, WI, USA). The spectrum (350 to 650 nm) of this extract was recorded using a Ati-Unicam UV2 spectrophotometer (Ati-Unicam, Cambridge, UK). Individual pigments were analyzed by using high-performance liquid chromatography (HPLC) with photodiode array detection.

2.3. Chromatography

A Waters modular liquid chromatographic system (Waters, Milford, MA, USA) equipped with two M510 pumps, a M996 photodiode array detector and a Rheodyne (Cotati, CA, USA) Model 7125 injector with a sample loop of 20 μl were used, along with a Millenium 2010 Chromatography Data Management system. A Kromasil 100 C_{18} (Teknokroma, Barcelona, Spain), 5 μm, 25 cm×4.6 mm I.D. column was used, and elution was carried out following a modification of the chromatographic program proposed by Strack et al. [15]. The program consisted of a 30-min linear gradient elution from solvent A (1% acetic acid in water) to 12% solvent B (1% acetic acid in acetonitrile) with a flow of 1 ml/min. In each analysis, 20 μl of the filtered extract was directly injected onto the chromatographic column. The identities of the different chromatographic peaks were confirmed by their visible spectral characteristics in comparison to standards and retention times.

2.4. Reagents

HPLC-grade methanol and acetonitrile (Romil, Loughborough, UK) were used in all experiments. Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Acetic acid was from Merck (Darmstadt, Germany). The eluents were degassed prior to use.

2.5. Influence of temperature on pigment stability

The heat stability of betacyanins was measured after treatment in a thermostatically controlled bath at 25, 50, 70 and 90°C. The samples were held at each temperature for specific times and then cooled immediately in an ice bath. Subsequently the absorption spectra of the solutions were recorded. Three replications of each experiment were carried out and the mean values and the standard deviations are shown.

3. Results and discussion

3.1. Extraction and spectroscopic analysis of pigments

Fig. 1 shows the visible absorption spectra (350–650 nm) of the methanolic extracts of the prickly pear fruits. The difference between the spectra is obvious. The spectrum of reddish purple fruits shows two peaks, one at 484 nm and the other at 533 nm, while the spectrum of yellow fruits, only has one absorption maximum at 483 nm. The absence of a peak at 533 nm would indicate that in these fruits betacyanins are to be found in a very low level and spectrophotometrically is very difficult to distinguish them from betaxanthins, which are present in a much higher concentration.

This preliminary analysis suggests that the external color of prickly pear fruits depends on the relative concentration of betacyanins (red pigments with maximum absorbance at around 535 nm) and betaxanthins (yellow pigments with maximum absorbance at around 480 nm). The concentration of pigments can be estimated using an extinction coefficient ($E^{1\%}_{1\ cm}$) of 1120 for betacyanins and 650 for betaxanthins [7,16]. The average amount obtained in the reddish purple prickly pear fruits was about 19 mg of betacyanins per 100 g of fresh pulp, and about 30 mg of betaxanthins per 100 g, a ratio of about 2:3. These results are similar to those reported by Forni et al. [7] where quantities of about 15 mg of betacyanins per 100 g of fresh pulp have been reported. In the yellow fruits, only betaxanthins were detected, with yield of about 25 mg per 100 g of fresh pulp. It has to be emphasized that the determination of the pigment contents according to the extinction coefficients is valid only for pure solutions and it is biased in the case of natural extracts.

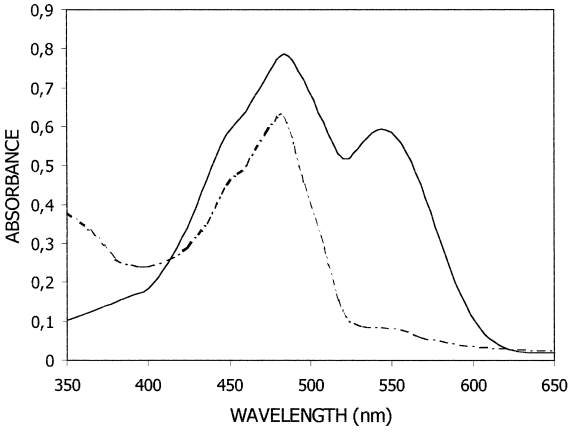

Fig. 1. Visible light absorption spectra of reddish purple (solid line) and yellow (dotted line) prickly pear fruits.

3.2. HPLC analysis of pigments

Since betacyanins and betaxanthins possess similar spectroscopic and chromatographic properties, HPLC is an invaluable means of separating and analyzing them. Tentative identification of these betalains can be deduced from their chromatographic behavior, and corroborative data may be provided by an analysis of their absorption spectra.

Fig. 2 shows the chromatographic pattern of the methanolic extract of reddish purple fruits. At 484 nm, two major peaks can be observed eluting at 16.2 min (peak 1) and 17.4 min (peak 2). When the same extract is monitored at 535 nm, a large peak with a retention time of 17.4 min is observed (peak 2). Peak 1 showed maximum absorbance (λ_{max}) at 484 nm

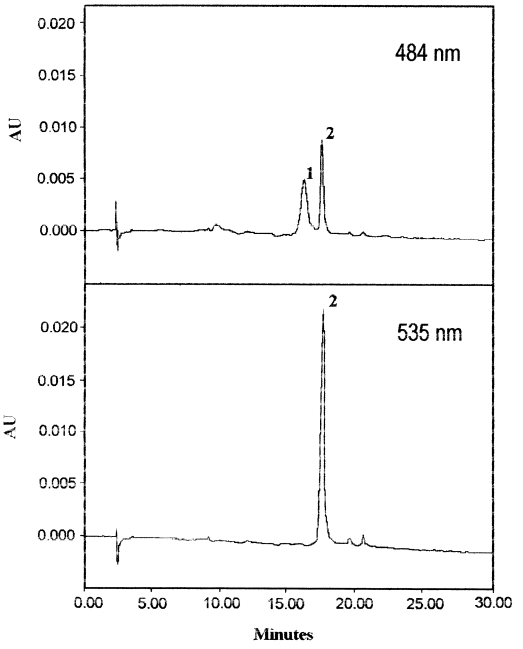

Fig. 2. HPLC chromatograms of betalain pigments from reddish purple prickly pear fruits. Peaks: 1=indicaxanthin; 2=betanin.

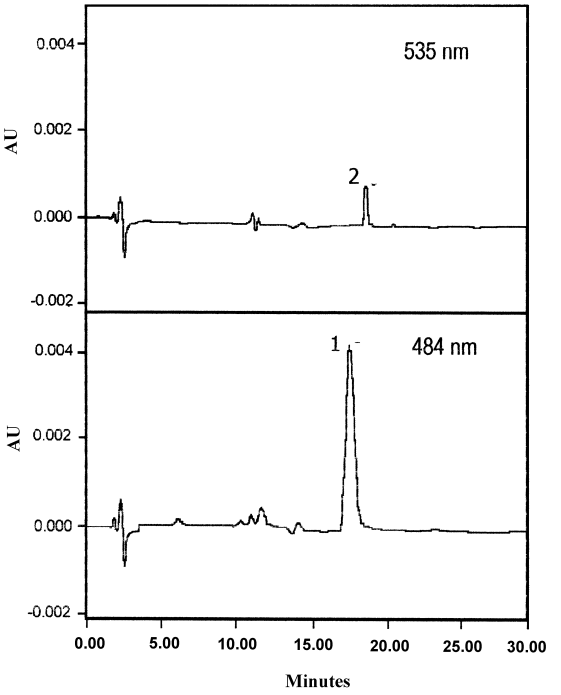

Fig. 3. HPLC chromatograms of betalain pigments from yellow prickly pear fruits. Peaks: 1=indicaxanthin; 2=betanin.

and peak 2 at 535 nm. From the respective retention times in comparison to standards, the spectral properties provided by the photodiode array detector and data reported by other researchers [6–8,16] peak 1 was identified as indicaxanthin and peak 2 as betanin.

The chromatograms at 484 and 535 nm of the methanolic extract of yellow fruits are shown in Fig. 3. When monitored at 484 nm only one peak appeared with the same retention time and spectral properties as peak 1 of Fig. 2, which would correspond to indicaxanthin. At 535 nm only a minor peak with the same retention time and λ_{max} as peak 2 of Fig. 2 was detected, which was identified as betanin. This would confirm the presence of betacyanins in the yellow prickly pear fruits, but at very low concentration. The contour plots of the chromatograms demonstrate the symmetry of all the peaks in the wavelength dimension, and point to the high degree of purity of the bands (Fig. 4). The chemical structures of betanin and indicaxanthin are shown in Fig. 5.

It is remarkable that indicaxanthin is the only betaxanthin found in the two *Opuntia ficus-indica* cultivars analyzed. Other researchers have reported other betaxanthins including vulgaxanthin, miraxanthin and portulaxanthin [17]. At the same time, betanin is the only betacyanin found in our studies, while neobetanin has been reported in an earlier study [15].

3.3. Pigment stability

The influence of temperature on pigment stability was determined in the extracts obtained from reddish purple prickly pear fruits to ascertain the potential use of these red pigments as a natural colorant. When the red extracts containing the betacyanin pigments were heated, the red color gradually disappeared. The absorbance at 535 nm diminished proportionally with the intensity of the heat treatment. At the same time, the absorbance at 484 nm increased slightly with an isosbestic point at 496 nm (Fig. 6). The existence of this isosbestic point suggests that the product with maximum absorbance at 484 nm is formed directly from the pigment with maximum absorbance at 535 nm through one chemical reaction [6]. A shoulder at 456 nm can be detected in the spectra where the thermodegradation of the initial red pigment is more intense. The quantitative data

Table 1
Heat stability of betacyanin pigment from reddish purple prickly pear fruits[a]

Temperature (°C)	Initial absorbance (535 nm)	Absorbance after 30 min (535 nm)	Thermodegraded pigment (%)
25	0.623±0.001	0.621±0.003	0.3±0.2
50	0.623±0.001	0.400±0.010	35.8±1.6
70	0.623±0.001	0.159±0.030	74.5±4.8
90	0.623±0.001	0.060±0.006	90.4±1.0

[a] Mean values±standard deviation of triplicate samples.

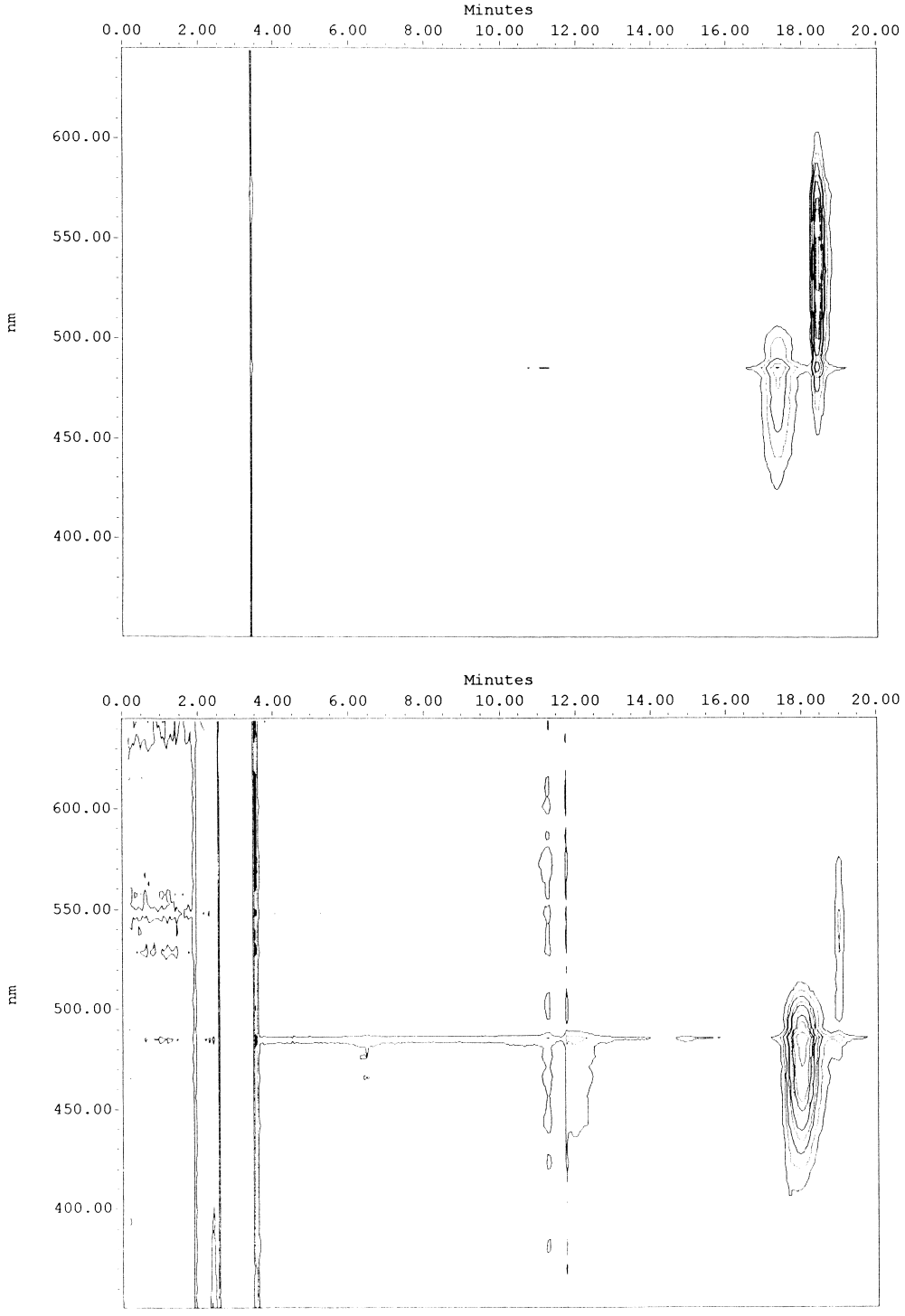

Fig. 4. Contour-plots of the chromatographic analysis of reddish purple (above) and yellow prickly pear fruits (below).

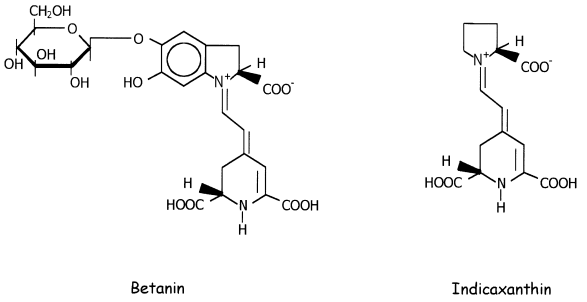

Fig. 5. Chemical structures of betanin and indicaxanthin.

referring to betacyanin losses during heating (Table 1) reveal that this pigment is very sensitive to temperature, treatment at 70°C for 30 min causing a loss of almost 75% of the initial value. When the temperature was raised to 90°C the loss increased to 90%.

4. Conclusion

Reversed-phase HPLC–photodiode array detection has been applied to the determination of betacyanin and betaxanthin pigments in *Opuntia ficus-indica* fruits grown in the southeastern Spain. Only the presence of betanin (betacyanin) and indicaxanthin (betaxanthin) was confirmed in the two cultivars analyzed. The estimation of the betacyanin pigment yield suggests that these fruits could be used as source of natural colorants. Quantitative data on red pigment losses during heating reveal the thermodegradation of these pigments at elevated temperatures.

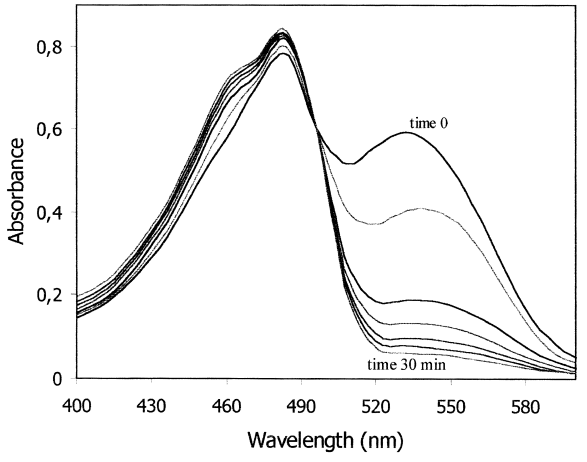

Fig. 6. Effect of the temperature on the visible light absorption spectrum of reddish purple prickly pear fruits. The different lines correspond to the spectra after exposure times at 90°C of 0, 5, 10, 15, 20, 25 and 30 min.

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