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PAPER

Production of a natural red pigment derived from *Opuntia* spp. using a novel high pressure CO_2 assisted-process

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Cactus pears (*Opuntia* spp.) have been identified to be an excellent source of betalain pigments which can be used as a red food colourant. In this work, pigments from cactus pear fruits were effectively extracted by High Pressure Carbon Dioxide assisted-acidified water extraction after a pre-treatment with CO_2 at 375 bar, 55 °C and 60 minutes. Different process conditions, namely pressure, temperature and volume ratio of (solid–liquid mixture)/(pressurized CO_2), were tested in order to model the extraction of betalains form prickly pears via the Response Surface Methodology. The best response was achieved at 100 bar, 40 °C and 20 % volume ratio of (solid–liquid mixture)/(pressurized CO_2). Under these conditions, the betalains yield was 89 ± 0.7 mg per 100 g of dried fruit, 83 % of the maximum extractable pigments from *Opuntia* spp. Fruits. Furthermore, *Opuntia* spp. extracts presented a vivid red colour with high antioxidant activity.

Introduction

In recent years, there is a growing interest in the development of natural colourants due to the health promoting effects of natural substances.[1] Furthermore, the safety of synthetic food colourants has been related to high levels of toxicity, allergic reactions, and carcinogenic potential.[2–7] Although synthetic dyes have lower production costs and greater stability, the European Union and the United States have restricted their use as food additives.[6,8] In this field, betalain pigments provide a natural alternative to synthetic red dyes.

Betalains are water-soluble vacuolar nitrogen-containing pigments, which are synthesized from the amino acid tyrosine into two structural groups, namely betacyanins with colour differences from purple to violet and betaxanthins with a range of colour from yellow to orange.[3,9–11] The added value of these pigments is increased owing to their double function as colourant and as antioxidant.[12–18]

The major commercial forms of betalains are produced from red beetroot juices (*Beta vulgaris* L.), available as either juice concentrates or powders, containing from 0.3 % to 1 % of pigment. This natural food colourant is classified as additive E-162 (EU) and 73.40 (FDA, USA).[2,19–23] However, red beet present some drawbacks including the poor colour spectrum and earthy-like flavour caused by geosmin, as well as high nitrate concentrations associated with the formation of carcinogenic nitrosamines.[11,19,21,24]

Cactus pears fruit have been identified to be a promising alternative betalainic crop covering a wide coloured spectrum from yellow to purple pigments. Cactus pear (*Opuntia* spp.) is a tropical or subtropical fruit tree, native to America, which grows in arid and semiarid regions.[5,25] The largest genus of the *Cactaceae* family is mainly used for fruit production. It enables rapid growth, good adaptation to poor soils and low requirement for water. Its fruit, cactus pear fruit or prickly pears, is a fleshy berry, varying in shape, size and colour has very tasty pulp full of seeds.[5,22,23,25,26] In contrast to red beetroot, cactus fruits do not contain geosmin and pyrazines that are responsible for the unpleasant pettiness of the former, represents lower risk for microbiological contamination, are highly flavoured, show adequate nutritional properties and contains interesting functional compounds.[19,20,22,23] Furthermore, the commercial exploitation of this fruit as an alternative source of food colourants may contribute to the sustainable development of the underdeveloped semi-arid regions.[2]

Betalains, which are synthesized in the cytoplasm and stored in vacuoles, are mainly extracted through conventional extractions.[5,22,27,28] These methods have several drawbacks, such as long extraction time, evaporation of a huge amount of solvent, stability problems, batch-to-batch variations, low selectivity and relative low yields.[6,8,29–34] Therefore, it is a key focus to develop novel extraction methods with faster extraction rates and higher betalain extraction yields. An

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efficient extraction should maximize betalain recovery with minimal degradation using environmentally friendly technologies.

Xu et al., 2010 and Santos & Meireles, 2011 have demonstrated that the explosive effect of High Pressure Carbon Dioxide (HPCD) besides inactivating microorganisms and enzymes could also strengthened the anthocyanin extraction from red cabbage and jabuticaba by its superior abilities in cell membrane modification, intracellular pH decrease, disordering of the intracellular electrolyte balance and removal of vital constituents from cells and cell membranes.[6,32] Up to date, there is no study on HPCD assisted-water extraction of betalains. However, Liu et al., 2008 showed that HPCD treatment of a red beet extract proved to be effective in inactivating the enzymes responsible for phenolic and betalains degradation. This treatment resulted in no significant change in the colour shade of that extract.[35]

The aim of this work was to obtain betalain-rich extracts from *Opuntia* spp. fruits. Within the present study, it was investigated for the first time, the feasibility of using a two-step process, HPCD pre-treatment followed by HPCD assisted-water extraction. Process variables were optimized, such as, pressure, temperature and volume ratio of (solid–liquid mixture)/(pressurized CO_2) ($R_{S\text{-}L/CO2}$ (%)) for the maximum recovery of betalains using acidified water. The yield obtained was compared with pressurized water extraction (PWE) and water extraction (WE). The red pigment was compared with commercial red beet extract regarding colour characteristics.

Experimental

Materials

Chemicals used for different extractions methodologies were: carbon dioxide 99.95% from Air Liquide (Lisbon, Portugal), methanol absolute 99.99% from Fisher Scientific (Waltham, MA, USA) and citric acid from Sigma- Aldrich (St Quentin Fallavier, France).

For phytochemical characterization: sodium carbonate (Na_2CO_3) was purchased from Sigma-Aldrich (St Quentin Fallavier, France), Folin-Ciocalteau reagent was acquired from Panreac (Barcelona, Spain) and gallic acid was purchased from Fluka (Germany).

Chemicals used for antioxidant activity assays were: 2`,2`-Azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid ($C_9H_8O_4$), cobalt fluoride tetrahydrate (CoF_2), hydrogen peroxide (H_2O_2) and picolinic acid ($C_6H_5NO_2$) from Sigma-Aldrich (St Quentin Fallavier, France) and iron chloride ($FeCl_3$) from Riedel-de-Haën (Seelze, Germany). Disodium fluorescein (FL) was from TCI Europe (Antwerp, Belgium).

Table 1. Actual values of the variables for the coded values.

Variable, factors, unit	Levels		
	-1	0	+1
Temperature, X_1 (°C)	40	55	70
Pressure, X_2 (bar)	100	175	250
$R_{S\text{-}L}/_{CO2}$, X_3 (%)	20	50	80

Reagents used for phosphate buffer solution (PBS) and sodium phosphate buffer solution (SPB) preparation included sodium chloride (NaCl), potassium chloride (KCl), monopotassium phosphate (KH_2PO_4) and sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$) from Sigma-Aldrich (St Quentin Fallavier, France) and sodium phosphate dibasic dehydrate ($Na_2HPO_4.2H_2O$) from Riedel-de-Haën (Seelze, Germany).

Opuntia spp. fruits preparation

Wild *Opuntia* spp. fruits were harvested by hand during October 2013. Fruits were collected from a plant growing in the South of Portugal (Algarve - Quarteira - N37°04.400, W008°06.100). All prickly pears fruits were harvested at comparable ripening stages (physiological maturity). For sample preparation, spikes were removed with a brush and *Opuntia* spp. fruits were freeze dried at -100 °C, in the absence of light, during 72 hours (FreeZone Plus 4.5 L Cascade Freezer Dry System, LABCONCO®, Kansas City, United States of America). The resulting dehydrated fruits were kept in a cold, dry and dark environment until further analyses.

Opuntia spp. fruits extraction procedures

High Pressure Carbon Dioxide assisted-water extraction

The Response Surface Methodology (RSM) was used to model the extraction of betalains from cactus pear fruits and optimize extraction conditions. The isolation of betalains through HPCD assisted-water extraction was carried out following a Central Composite Face-Centered Design (CCFC) as a function of three factors: pressure, temperature and volume ratio of (solid-liquid mixture)/(pressurized CO_2) ($R_{S\text{-}L/CO2}$). The CCFC is an experimental design used to achieve maximal information about a process from a minimum number of trials. The selected variables were tested simultaneously, using CCFC that enable to find interactions between the variables which cannot be identified with classical approaches.

From the literature [6,32] and some preliminary experimental results (data not shown), process variables and their ranges (extraction pressure (100 - 250 bar), extraction temperature (40 - 70 °C) and volume ratio of (solid-liquid mixture)/(pressurized CO_2) (20 - 80 %) were chosen (Table 1). After selection of independent variables and their ranges, experiments were established based on a CCFC design and each independent variable was coded at three levels between -1, 0 and +1. Taking into account the determined factor range to the extraction pressure (100 - 250 bar), the values 100 bar (level -1), 175 bar (level 0) and 250 bar (level +1) were selected. The same levels were defined for the temperature and $R_{S\text{-}L/CO2}$ according to Table 1. A total of 17 assays including three replicates of the center points were generated (Table 2).

The extractions were carried out in a supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SFE-500F-2-C50). Before each extraction, a High Pressure Carbon Dioxide (HPCD) pre-treatment of dried *Opuntia* spp. fruits was performed. A given weight (4.2-11.1 g) of dried *Opuntia* spp. fruits were placed in the extraction vessel, which was pre-heated to 55 °C and then pressurized by CO_2 until 375 bar for 60 minutes. These conditions were chosen accordingly to Liu et al., 2008.[35]

Table 2. The CCFC design for the three independent variables.

Experiment number	Temperature, X_1 (°C)	Pressure, X_2 (bar)	$R_{S-L/CO2}$, X_3 (%)
1	40 (-1)	100 (-1)	20 (-1)
2	40 (-1)	100 (-1)	80 (+1)
3	40 (-1)	250 (+1)	20 (-1)
4	40 (-1)	250 (+1)	80 (+1)
5	70 (+1)	100 (-1)	20 (-1)
6	70 (+1)	100 (-1)	80 (+1)
7	70 (+1)	250 (+1)	20 (-1)
8	70 (+1)	250 (+1)	80 (+1)
9	70 (+1)	175 (0)	50 (0)
10	70 (+1)	175 (0)	50 (0)
11	55 (0)	100 (-1)	50 (0)
12	55 (0)	250 (+1)	50 (0)
13	55 (0)	175 (0)	20 (-1)
14	55 (0)	175 (0)	80 (+1)
15 (C)	55 (0)	175 (0)	50 (0)
16 (C)	55 (0)	175 (0)	50 (0)
17 (C)	55 (0)	175 (0)	50 (0)

At the end of each pre-treatment, pressure was slowly release and a specific volume (84-222 mL) of acidified water pH 5.0 by citric acid (ratio 1:20, w/v) was added to the extraction vessel which was pre-heated to the required temperature (40-70 °C). Liquid CO_2 was fed in the extraction vessel using a TharSFC P-50 high pressure pump (Thar Technology, Pittsburgh, PA, USA). The vessel was pressurized with carbon dioxide until reaching the required pressure (100-250 bar) and held for 30 minutes. After this extraction time, the depressurization was performed by releasing CO_2 into the atmosphere. The resulting extracts were kept in a cold, dry and dark environment until further analyses.

Pressurized Water Extraction (PWE)

Pressurized water extraction was carried out in the extractor previously mentioned (Thar Technology, Pittsburgh, PA, USA, model SFE-500F-2-C50) with the same solvent at the same extraction pressure and temperature.

The extraction vessel was filled with about 10 g of dried *Opuntia* spp. fruits and laboratory glass beads were placed on both endings of the cell, in order to achieve a uniform distribution of the solvent flow. Acidified distilled water pH 5.0 by citric acid was delivered to the extraction vessel using a TharSFC P-50 high pressure pump (Thar Technology, Pittsburgh, PA, USA) until the desired pressure 100 bar. The solvent was preheated on a heat exchanger to a temperature of 40 °C. The pressure on the extraction vessel was maintained by an automated back pressure regulator (TharSFC ABPR, Thar Technology, Pittsburgh, PA, USA), which was located between the extraction vessel and the first fraction collector, with a total solvent flow rate of 10 g/min. The resulting extract was kept in a cold, dry and dark environment until further analyses.

Water Extraction (WE)

An extraction under atmospheric pressure was performed as a control with acidified water pH 5.0 by citric acid (ratio 1:20, w/v), for 30 minutes at 40 °C. The resulting extracts were kept in a cold, dry and dark environment until further analyses.

Conventional Solvent Extraction

In order to quantify the maximum amount of extractable pigments from *Opuntia* spp. fruits, complete extraction of betalains was conducted by mixing 0.1 g of freeze dried fruits with 10 mL of aqueous methanol (ratio 1:1, v/v) for 30 minutes at room temperature.[36] The extract was centrifuged at 5000 RCF (relative centrifugal force) for 10 minutes (MIKRO 220 R, Hettich Zentrifugen, Tuttlingen, Germany). The supernatant was recovered after centrifugation and the residue was extracted again with aqueous methanol until absence of colour was obtained. All extracts were combined and kept in a cold, dry and dark environment until further analyses.

***Opuntia* spp. fruit extracts characterization**

Total Betalain Content (TBC)

The total betalain content of the *Opuntia* spp. fruit extracts was calculated according to Guzmán-Maldonado et al., 2010.[36] All extracts were spectrophotometrically measured at 476, 538, and 600 nm. Betacyanins and betaxanthins were determined with Nilsson equations:

% Betacyanins = ([a/1129] × DF × 100),

% Betaxanthins = ([y/750] × DF × 100),

where $a = 1.095(A_{538} - A_{600})$,

$y = A_{476} - (A_{538} - a) - (a/3.1)$ and

DF = dilution factor.

The total betalain content was expressed as the sum of betacyanin and betaxanthins content. The results were presented as mg of betalains per 100 g of dried fruit, expressed as a mean of triplicates.

Total Phenolic Content (TPC)

The total concentration of phenolic compounds present in *Opuntia* spp. fruit extracts was determined according to the modified Folin-Ciocalteau colorimetric method as previously described by Serra et al., 2008.[37,38] The results were presented as mg of gallic acid equivalents (GAE) per 100 g of dried extract and were expressed as a mean of eight replicates.

Antioxidant activity

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was carried out by the method of Huang et al., 2002 modified for the FLx800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by Feliciano et al., 2009.[39,40] This assay measured the ability of the antioxidant species in the sample to inhibit the oxidation of disodium fluorescein (FL) catalysed by peroxyl radicals generated from AAPH. The final ORAC values were calculated by the EC (Effective Concentration) method to diminish the impact of the dilution effect as described by Bolling et al., 2012.[41] The results were presented as µmol of the trolox

equivalent antioxidant capacity (TE) per 100 g of dried extract and were expressed as a mean of eight replicates.

Hydroxyl radical adverting capacity (HORAC)

The HORAC assay was based on a previously reported method modified for the FLx800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA).[42] This assay evaluates the hydroxyl radical prevention capacity of a sample using fluorescein as a probe. The final HORAC values were calculated by the EC (Effective Concentration) method to diminish the impact of the dilution effect as described by Bolling et al., 2012.[41] Data were expressed as µmol of the caffeic acid equivalent antioxidant capacity (CAE) per 100 g of dried extract. Results were presented as a mean of eight replicates.

Hydroxyl radical scavenging capacity (HOSC)

The HOSC assay was performed according to Moore et al., 2006 and adapted for the FLx800 fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA).[43] This assay evaluates the hydroxyl radical scavenging capacity of a sample using fluorescein as a probe and a classic Fenton reaction with Fe (III) and H_2O_2 as a source of hydroxyl radicals. The final HOSC values were calculated by the EC (Effective Concentration) method to diminish the impact of the dilution effect as described by Bolling et al., 2012.[41] Data were expressed as µmol of the trolox equivalent antioxidant capacity (TE) per 100 g of dried extract. Results were presented as a mean of eight replicates.

Spectral and colour analyses

The visible spectra (400-700 nm) of the betalain-rich extract was recorded using an UV-Vis spectrometer (Genesys 10uv, Thermo Spectronic, Waltham, MA, USA).

The colour strength of all extracts was determined as the absorbance units at the maximum absorption wavelength of a 1 % (v/v) solution.[19]

The colour of *Opuntia* spp. extract were assessed by CIElab method using a Minolta Colorimeter CR-200 (Osaka, Japan) described using 3 attributes or specific qualities of visual sensation: tonality, luminosity and chromatism.

CIELab colour or space system is based on a sequential or continuous Cartesian representation of 3 orthogonal axes: L*, a* and b*. Coordinate L* represents clarity (L*=0 black and L*=100 colourless), a* green/red colour component (a*>0 red, a*<0 green) and b* blue/yellow colour component (b*>0 yellow, b*<0 blue).

C* is the chroma or colour purity and h° refers to the hue angle of tone and indicates the sample's colour (0° or 360°=red, 90°=yellow, 180°=green, and 270°=blue). C* was determined according to the expression $C^* = [(a^*) + (b^*)^2]^{1/2}$ and h° according to the expression h°=arctan(b*/a*).

The colour parameters were expressed as a mean of triplicates. These values were then converted to RGB (Red, Green, and Blue colour values), using the software OpenRGB (Logicol).

Experimental design analysis/statistical analysis

The results of the CCFC, concerning the total betalain content, total phenolic content, antioxidant activity (ORAC, HORAC and HOSC), colour strength and L* value were analysed using the software Statistica™, version 5, from Statsoft (Tulsa, USA). Both linear and quadratic effects of each factor under study, as well as their interactions were calculated. Their significance was evaluated by analysis of variance. A surface, described by a second-order polynomial equation, was fitted to each set of experimental data points. First- and second-order coefficients of the polynomial equations were generated by regression analysis. The fit of the models was evaluated using the determination coefficients (R^2) and adjusted R^2 (R_{adj}^2).

Results and discussion

Aiming at producing a red natural colourant from *Opuntia* spp. fruits a two-step process was developed. The first step consisted in a High Pressure Carbon Dioxide (HPCD) pre-treatment of dried prickly pears at 375 bar, 55 °C during 60 min. These conditions were chosen accordingly to Liu et al., 2008 for avoiding enzymatic degradation.[35] Moreover, this HPCD pre-treatment was applied in order to disrupt the cell vacuoles upon the depressurization of CO_2, making betalains more available. In a second step High Pressure Carbon Dioxide assisted-water extraction was explored. RSM was applied aiming at maximizing the extraction yield and stability of betalains. In order to define the extraction media, some previous studies on betalains extraction from *Opuntia* spp. were taken into account. Castellar et al., 2003 and Sanchez-Gonzalez et al., 2013 concluded that maximum stability of the *Opuntia* spp. pigments was achieved at pH 5.[9,34] Consequently, the water used in all extractions experiments was acidified until pH 5 using citric acid. The resulting extracts were evaluated in terms of betalains yield, phytochemical composition, antioxidant capacity and colour analyses.

Modelling of betalain extraction through HPCD assisted-water extraction

The HPCD assisted-water extraction experiments were carried out according to the CCFC previously described. The obtained results, i.e. the betalains yield, total phenolic content, antioxidant activity (ORAC, HORAC and HOSC), colour strength and L* value are shown in Table 3. These results were used to estimate both, linear and quadratic effects of the variables and also their linear interactions. For total phenolic content, ORAC, HOSC, colour strength and L*, a lack of fit of the polynomial models exhibited by low values of R^2 and R_{adj}^2 was observed.

The response surfaces (Figure 1) fitted to betalains yield can be described by second-order polynomial models as a function of pressure, temperature and $R_{S\text{-}L/CO2}$ (Table 4). In these response surface models, the significant effects $p < 0.05$ and those having confidence range smaller than the value of the effect, or smaller than the standard deviation (data not shown), were included in the model equations of these surfaces. It is better to accept factor with values higher than 0.05 rather than to take the chance of missing an important factor.[44] The good values for both R^2 and R_{adj}^2 of these models (Table 4) suggest a close agreement between the experimental

data and the theoretical values predicted by the model. About 82 % or 74 %

Table 3. Summary of the experimental results.

Experiment number	HPCD assisted-water extraction			Phytochemical composition			Antioxidant activity			Colour properties						
	T	P	$R_{s\text{-}L/CO2}$	TBC		TPC	ORAC	HORAC	HOSC	Colour strength	L*	a*	b*	C*	h°	RGB colour
	°C	bar	%	mg/100g dried *Opuntia*	mg/100g dried extract	mg/100g dried extract	µmol TE/100g dried extract	µmol CAE/100g dried extract	µmol TE/100g dried extract							
With HPCD pre-treatment (375 bar, 55°C, 60 min)																
1	40	100	20	88.7 ± 0.7	190.6 ± 4.4	929 ± 22	11104 ± 886	9423 ± 995	12828 ± 865	5.03 ± 0.20	28.28 ± 0.05	62.41 ± 0.09	24.61 ± 0.01	67.08	0.38	
2	40	100	80	85.0 ± 0.7	184.8 ± 4.2	930 ± 4	11697 ± 790	11417 ± 751	15762 ± 901	3.95 ± 0.08	30.26 ± 0.02	64.36 ± 0.06	20.51 ± 0.04	67.57	0.31	
3	40	250	20	79.8 ± 0.6	180.2 ± 4.1	890 ± 11	12109 ± 844	9666 ± 810	9605 ± 994	4.29 ± 0.13	32.76 ± 0.08	66.23 ± 0.10	21.34 ± 0.08	69.58	0.31	
4	40	250	80	74.6 ± 0.6	179.3 ± 4.1	934 ± 8	12585 ± 1762	11244 ± 670	12542 ± 1236	4.18 ± 0.05	31.01 ± 0.04	64.57 ± 0.06	19.35 ± 0.04	67.41	0.29	
5	70	100	20	73.9 ± 0.6	175.9 ± 4.0	901 ± 39	12828 ± 1548	9327 ± 839	14613 ± 1450	4.79 ± 0.03	30.39 ± 0.03	63.22 ± 0.06	21.73 ± 0.01	66.85	0.33	
6	70	100	80	72.8 ± 0.6	175.1 ± 4.0	864 ± 4	10271 ± 1643	9416 ± 1077	12250 ± 864	4.38 ± 0.05	33.59 ± 0.00	67.30 ± 0.10	24.20 ± 0.04	71.55	0.35	
7	70	250	20	76.2 ± 0.6	173.9 ± 4.0	962 ± 43	12653 ± 1325	9084 ± 1168	11857 ± 940	4.28 ± 0.13	30.32 ± 0.03	63.80 ± 0.02	26.88 ± 0.03	69.24	0.40	
8	70	250	80	81.6 ± 0.7	177.5 ± 4.1	929 ± 9	10849 ± 1479	9558 ± 725	14372 ± 1027	4.42 ± 0.06	33.96 ± 0.01	66.01 ± 0.01	23.61 ± 0.01	70.11	0.34	
9	40	175	50	79.7 ± 0.6	170.5 ± 3.9	890 ± 10	10642 ± 831	8499 ± 897	8659 ± 1092	4.99 ± 0.10	34.76 ± 0.03	67.44 ± 0.08	19.56 ± 0.02	70.21	0.28	
10	70	175	50	77.2 ± 0.6	179.3 ± 3.9	960 ± 37	12809 ± 842	11003 ± 841	13292 ± 1119	4.34 ± 0.06	33.16 ± 0.03	66.57 ± 0.01	25.24 ± 0.03	71.19	0.36	
11	55	100	50	75.4 ± 0.6	167.0 ± 3.8	969 ± 49	12829 ± 926	10330 ± 1150	10245 ± 934	4.62 ± 0.05	33.72± 0.03	67.64 ± 0.01	20.99 ± 0.04	70.82	0.30	
12	55	250	50	76.0 ± 0.6	174.8 ± 4.0	937 ± 13	11428 ± 1151	8576 ± 1062	12163 ± 807	4.59 ± 0.04	30.22 ± 0.04	64.93 ± 0.01	23.48 ± 0.04	69.05	0.35	
13	55	175	20	76.2 ± 0.6	173.6 ± 4.0	934 ± 39	11182 ± 839	9108 ± 452	11401 ± 635	4.85 ± 0.23	28.69 ± 0.04	62.38 ± 0.04	24.75 ± 0.02	67.11	0.38	
14	55	175	80	74.8 ± 0.6	171.8 ± 3.9	872 ± 33	12862 ± 1054	8640 ± 688	10575 ± 1570	4.28 ± 0.07	31.97 ± 0.02	64.60 ± 0.10	19.99 ± 0.00	67.62	0.30	
15 (C)	55	175	50	81.1 ± 0.6	181.3 ± 4.1	933 ± 30	10220 ± 1212	7182 ± 814	12174 ± 882	4.07 ± 0.09	32.78 ± 0.02	67.35 ± 0.01	21.79 ± 0.02	70.79	0.31	
16 (C)	55	175	50	86.8 ± 0.7	187.8 ± 4.3	982 ± 23	11476 ± 1460	8203 ± 616	11689 ± 848	3.71 ± 0.11	30.81 ± 0.04	65.17 ± 0.08	22.82 ± 0.08	69.04	0.34	
17 (C)	55	175	50	85.1 ± 0.7	184.6 ± 4.2	929 ± 31	10209 ± 1466	7766 ± 873	10248 ± 1440	4.51 ± 0.12	30.52 ± 0.02	64.93 ± 0.04	24.55 ± 0.04	69.41	0.36	
Without HPCD pre-treatment																
HPCDAWE	40	100	20	64.6 ± 0.3	175.6 ± 0.8	742 ± 14	10447 ± 1323	8159 ± 838	11848 ± 1019	3.77 ± 0.03	53.30 ± 0.04	80.55 ± 0.02	10.00 ± 0.06	81.16	0.12	
PWE	40	100		30.4 ± 1.2	164.2 ± 6.3	787 ± 22	11995 ± 1417	6834 ± 751	13164 ± 1078	1.67 ± 0.07	41.67 ± 0.02	75.74 ± 0.06	5.29 ± 0.05	75.92	0.07	
WE	40	1		45.4 ± 2.0	146.6 ± 4.9	818 ± 3	10205 ± 1050	7611 ± 709	11042 ± 1306	2.06 ± 0.03	36.33 ± 0.04	59.37 ± 0.02	15.25 ± 0.05	61.29	0.25	

HPCD, High Pressure Carbon Dioxide; **HPCDAWE**, High Pressure Carbon Dioxide Assisted Water Extraction; **PWE**, Pressurized Water Extraction; **WE**, Water Extraction; **TBC**, Total Betalain Content; **TPC**, Total Phenolic Content; **ORAC**, Oxygen radical absorbance capacity; **HORAC**, Hydroxyl radical adverting capacity; **HOSC**, Hydroxyl radical scavenging capacity.

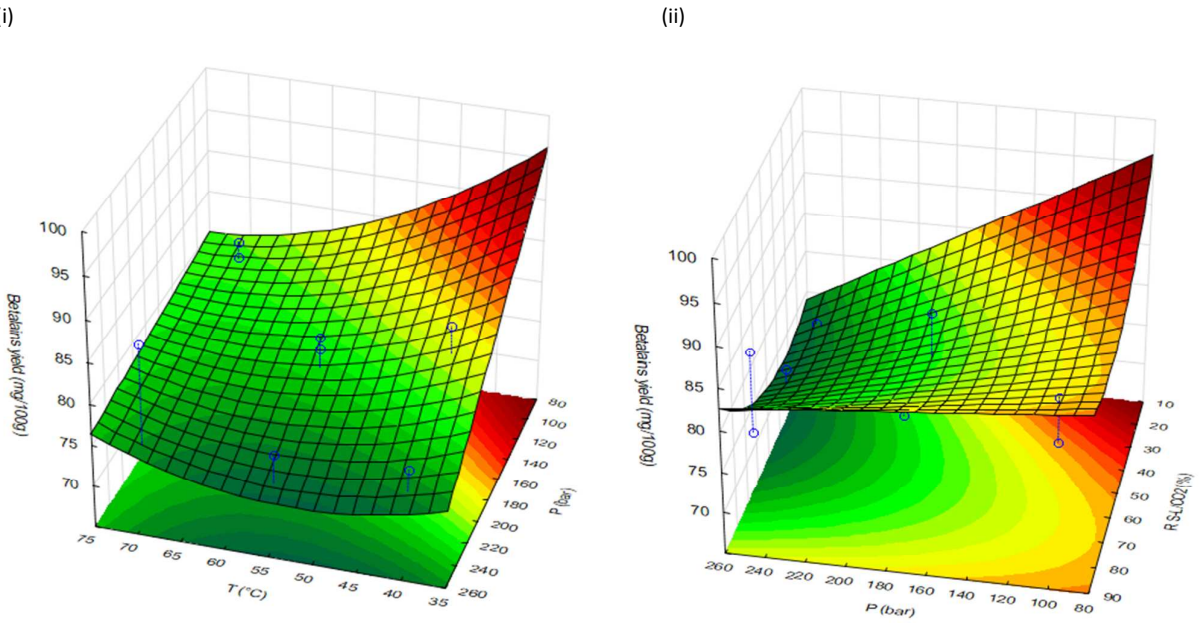

Figure 1. Response surfaces fitted to the betalains yield as a function of (i) pressure and temperature and (ii) pressure and $R_{S\text{-}L/CO2}$.

of the observed results concerning the betalains yield and HORAC are explained by the present model (see ESI). However, no optimum conditions were observed in the response surface for the betalains extraction. Therefore, only the identification of the region corresponding to the best response can be achieved.

The analysis of the data showed that the recovery of betalains was negative affected ($p < 0.05$) by the extraction pressure within the tested range (100 - 250 bar). Accordingly, the lower pressure tested (100 bar) led to an increase in betalains yield. This effect was more pronounced at lower temperatures (40 °C) and lower $R_{S\text{-}L/CO2}$ (20 %).

In addition, the recovery of betalains was significant affect ($p < 0.05$) by some interactions between factors, pressure with temperature and pressure with $R_{S\text{-}L/CO2}$ (Figure 1). With simultaneous increase of pressure and temperature values, the betalains yield decreased. This result may be explained by the negative impact of higher pressure and temperature on the pigments stability during the extraction process. This effect was also reported by Santos & Meireles, 2011 for other pigment.[6] When the pressure and $R_{S\text{-}L/CO2}$ values decreased the recovery of betalains increased. This effect can be explained by the increase of the volume of pressurized CO_2 (lower $R_{S\text{-}L/CO2}$), which possibly plays a crucial role during the betalains extraction.

The best response can be achieved at 100 bar, 40 °C and 20 % volume ratio of (solid–liquid mixture)/(pressurized CO_2). Under these conditions, the betalains yield was 89 ± 0.7 mg per 100 g of dried fruit.

The repeatability (coefficient of variation) of the extraction process through HPCD assisted-water extraction was 4 %, taking into account three experiments of the design (center points).

Available literature on *Opuntia* spp. fruits illustrates a high variation of betalains content. The total pigment content depends on the respective species and clone investigated and may range from 5 – 110 mg/100 g.[9,14,34] Our results agree with those findings reported in literature (17.8 mg/100 g fresh fruit).

The effect of High Pressure Carbon Dioxide on the two-step extraction process

The effect of HPCD on betalains extraction was also studied throughout the HPCD pre-treatment and HPCD assisted-water extraction steps. A comparison of extraction percentage of betalains obtained with two-step extraction process, HPCD assisted-water extraction without pre-treatment, pressurized water extraction (PWE) and water extraction (WE) with respect to the maximum extractable pigment (107 ± 2.12 mg of betalains/100 g dried fruit) is shown in Figure 2.

Table 4. Model equations for the response surfaces fitted to the values of betalains yield (BY), and HORAC, as a function of Pressure (A), Temperature (B) and $R_{S\text{-}L/CO2}$ (C), and respective R^2 and R_{adj}^2.

Polynomial model equations	R^2	R_{adj}^2
$BY = 163.6 - 0.237A - 1.701B + 0.009B^2 - 0.634C + 0.003C^2 + 0.003AB + 0.001AC + 0.003BC$	0.817	0.581

$HORAC = 8964 + 0.306B^2 – 40.57C -0.891C^2 – 0.204AB + 0.232AC$	0.736	0.617

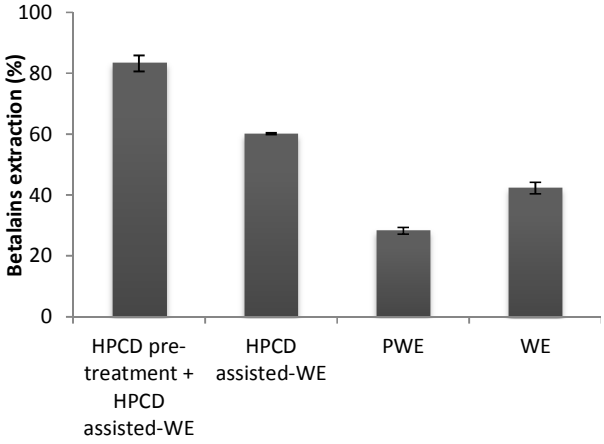

Figure 2. Yield of betalain relatively to the content obtained in the conventional solvent extraction.

The greatest extraction percentage of betalains was obtained with two-step extraction process, with extraction percentage of 83 % of the maximum extractable pigments from *Opuntia* spp. fruits (Figure 2). The amount of pigments extracted by HPCD assisted-water extraction, PWE and WE were lower (28 – 60 %). The promising effect of the use of pressurized CO_2 for betalains extraction seems irrefutable when the results obtained using this technique were compared to that obtained using other extraction methods. Comparing WE with PWE and HPCD assisted-water extraction at same extraction conditions (temperature, solvent and ratio S-L) it was possible to conclude that the last was more efficient in extracting betalains from *Opuntia* spp. fruits. In addition, the HPCD pre-treatment before the HPCD assisted-water extraction has also enhanced the extraction efficiency.

According to Santos & Meireles, 2011 and Xu et al., 2010, there are five possible forms associated with CO_2 in a HPCD assisted-water extraction, including supercritical CO_2, carbonic acid and its dissociated products (H^+, HCO_3^- and CO_3^{-2}). These different forms might play different roles in the HPCD assisted-water extraction of betalains. Firstly, supercritical CO_2 combines high diffusivity of gas with solvent strength of liquids with non-polar and lipophilic properties to dissolve phospholipid layer of cell membranes, improving the penetration of water into the cellular matrix and efflux of betalains from cell vacuoles to the outside of the cell. Secondly, the generation of in situ carbonic acid, when the CO_2 is added into HPCD assisted-water extraction system, decrease pH. This leads to a positive impact on betalains extraction and stability from *Opuntia* spp. fruits. Finally, the explosive effect during the rapid CO_2 depressurization causes disruption of cell vacuoles, making betalains more available, thus enhancing extraction efficiency.[6,32]

In addition, the effect of pressurized carbon dioxide has been reported to inactivate microorganisms and inhibit enzyme activity (polyphenoloxidase and peroxidase), which are responsible for betalains degradation.[45] Furthermore, the absence of oxygen in the extraction system is other advantage of this methodology because the presence of oxygen is one of the factors that affect betalains stability.[6,32] Overall, these advantages have contributed for the higher yields of betalains using HPCD.

Opuntia spp. fruit extracts characterization

Phytochemical content and functional properties

As the quality of an extract is related to its phytochemical composition, and then, to its functional properties, the *Opuntia* spp. fruit extracts obtained through HPCD assisted-water extraction were analysed in terms of the total phenolic content and antioxidant capacity (ORAC, HORAC and HOSC). The results are presented in Table 3.

Regarding the total phenolic content, the values varied between 864 ± 4.13 and 982 ± 23.0 mg GAE/100 g dried extract. Among all extracts the ORAC, HORAC and HOSC varied between 10209 ± 926 and 12862 ± 1212 µmol TE/100 g dried extract, 7182 ± 688 and 11417 ± 873 µmol CAE/100 g dried extract, and 8659 ± 901 and 15762 ± 1440 µmol TE/100 g dried extract.

The antioxidant activity results are in line with that has been obtained by other authors. Several works have demonstrated the potent antiradical scavenging activity of betalains in vitro.[13,15,26,46] It was shown that betacyanins i.e. betanin acts as a scavenger of reactive oxygen species (DPPH-, galvinoxyl-, superoxide- and hydroxyl radicals).[16] Furthermore, other authors have proved that betalains prevent active oxygen-induced and free radical-mediated oxidation of biological molecules.[12,15,17,18]

Spectral and colour analyses

To assess the commercial applicability of the produced *Opuntia* spp. fruit extracts their colour properties were determined and compared with a commercial liquid concentrated extract.

Firstly, a spectrophotometric study for all extracts was performed by normalizing to an absorbance of 0.70 ± 0.05 at 535 nm, the betanin λ_{max} value (See ESI). It can be seen that *Opuntia* spp. fruit extract showed single maximum wavelengths at 535 nm and presented a symmetrical spectrum. These results indicated that *Opuntia* spp. fruit extract spectrum is characteristic of a single-composition (betanin/isobetanin).[19]

Opuntia spp. fruits only contain the red pigments betanin and isobetanin, which are the compounds present in the additive, red beet (E-162). These characteristics make cactus pear a promising source of betacyanin pigments, which would be suitable for food applications.

The colour parameters of the Opuntia spp. extracts was measured using CIELab method. The results are presented in Table 3. From the results, lightness ranged from the L* = 28.7 (best response) to the L*= 34.8. All the samples had positive a* values, as expected from their red colour ranging from a* =

62.4 to a* = 67.6. It was observed positive values of b* parameter (blueness–yellowness) ranging from b* = 19.3 to b* = 26.9. This means that all samples had a more yellow colour than blue. Chroma, which expresses the brilliance or purity of a colour, was similar in all extracts. The hue angle, which indicates the tonality, ranged between 0.28 and 0.40, as predictable from their red colour.

Table 5. Colour properties of the produced *Opuntia* spp. fruit extract and the commercial red beet concentrate.

	Colour strength	Colour parameters						
		L*	a*	b*	C*	h°	ΔE*	RGB colour
Opuntia spp. fruit extract	5.0 ± 0.2	28.7 ± 0.0	62.4 ± 0.1	24.6 ± 0.0	67.1	0.38	0.0	
Red beet concentrate [19]	5.2 ± 0.1	69.5 ± 0.1	57.9 ± 0.2	-1.0 ± 0.0	57.9	359	8.4	

Finally, colour proprieties of the selected *Opuntia* spp. fruit extract were compared with those of a commercial red food colourant (Table 5).

Red beet pigment has been extensively commercialized as a food colourant. E-162 and 73.40 are the commercial codes for red beet pigment in Europe and U.S.A., respectively.

According to the results, the *Opuntia* spp. extract presented high colour strength like the commercial form. The total colour difference value (ΔE*) was 8.4, more than 5 units of difference indicate that human eye is capable of distinguishing *Opuntia* spp. fruit extract from the red beet concentrate. *Opuntia* spp. fruit extract had the lowest value of lightness. Both had positive a* values due to their red colour. Greater dispersion was observed in the b* parameter, the *Opuntia* spp. fruit extract presented more yellowness colour than blueness. The brilliance or purity of a colour was highest in *Opuntia* spp. fruit extract and, according to its hue value, was the reddest colourant. Therefore, *Opuntia* spp. fruit extract, with its pleasant flavour, could be considered as a promising natural betalain food colourant.

Conclusions

A novel high pressure CO_2-assisted process was proposed in this work to promote red pigment recovery from *Opuntia* spp. fruits. The best operating conditions to obtained betalains-rich extracts were achieved at 100 bar, 40 °C and 20% $R_{S\text{-}L/CO2}$, after high pressure CO_2 pre-treatment at 375 bar, 55 °C and 60 min. Under these optimized conditions, the betalain extraction yield was 2-fold increased when comparing to conventional water extraction. This process provided betalain-rich extract with a vivid red colour and high colour strength and significant antioxidant activity (12862 ± 1212 µmol TE/100 g, 11417 ± 873 µmol CAE/100 g and 15762 ± 1440 µmol TE/100 g dried extract, for ORAC, HORAC and HOSC, respectively). Consequently, the *Opuntia* spp. fruit extract described in this study represents a natural red pigment alternative to red beet extracts with intensive reddish colour and improved sensory properties, without geosmin and pyrazines.

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