Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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A way to prepare a liposoluble natural pink colorant

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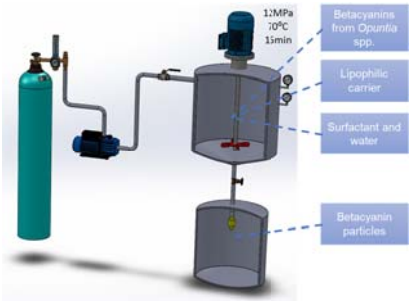
Graphical Abstract:

The modelling and development of a lipophilic natural pink colorant derived from *Opuntia* spp. was studied using a green technology - PGSS® (Particles from Gas-Saturated Solutions).



Abstract:

Cactus pears (*Opuntia* spp.) have been identified a promising betalainic crop covering a wide colour spectrum from yellow to purple pigments. The aim of this study was to exploit the use of Supercritical Fluid Technology to develop lipidic particles of betalain-rich extract derived from *Opuntia* spp. fruits to be further incorporated in food matrices. A natural extract was encapsulated by PGSS® (Particles from Gas Saturated Solutions) technique into glyceryl monoostearate, using a surfactant (Polyglyceryl-3 Polyricinoleate) and water. Different process conditions, namely pressure (9-23MPa), temperature (57-73^{0}C) and equilibrium time (5-55min) were tested in order to model the encapsulation of betalains *via* Response Surface Methodology (RSM), following a Central Composite Rotatable Design (CCRD). Results showed that temperature and pressure were the parameters that most affected the encapsulation of the natural pigment and the highest betacyanin encapsulation was achieved lower pressures. This effect was more pronounced at higher temperatures and lower equilibrium times. At these conditions, *Opuntia* spp. particles presented 64.4 mg/100g of betacyanins and high antioxidant capacity. Additionally, the particles with higher betacyanin content, showed an average particle size of 10 µm and a melting point between 57 and 65^{0}C. When compared with the *Opuntia* dried extract, lipidic particles contributed to a better homogenization of the pink colour after incorporation in ice cream.

Keywords: natural colour; betalains; *Opuntia* spp.; Supercritical Fluid Technology; PGSS®; lipophilic form.

Introduction

The use of natural pigments instead of synthetic colorants is receiving growing interest in the food industry due to the health promoting effects of natural substances. Recently in response to this trend (the use of natural pigments in the natural dyeing), healthy functional foods, cosmetic products for human health and safety have been gradually expanded[1-3].

Betalains are water-soluble nitrogen-containing pigments, which are synthesized from the amino acid tyrosine into two structural groups, namely the red-violet betacyanins and the yellow–orange betaxanthins[1-4]. They are present in most plants belonging to the order *Caryophyllales*, where they fulfil the role of anthocyanins. These pigments have attracted much attention because of their bioactivities, which range from an antioxidant capacity to the chemoprevention of cancer[5-8]. Betalains have several applications in foods such as desserts, confectionary, dairy and meat products[5-9]. The major commercial forms are produced from red beetroot juices (*Beta vulgaris L.*), available as either juice concentrates or powders, containing from 0.3% to 1% of pigment[4-9]. These extracts present some drawbacks including the poor colour spectrum and earthy-like flavour caused by geosmin and high nitrate concentrations associated with the formation of carcinogenic nitrosamines[3-5].

In this field, cactus pears (*Opuntia* spp.) have been identified to be a promising betalainic crop covering a wide coloured spectrum from yellow to purple pigments. This crop do not contain geosmin and pyrazines that are responsible for the unpleasant taste. Moreover, it represents lower risk for microbiological contamination, it is highly flavoured, it shows adequate nutritional properties and it contains interesting functional compounds[3-8]. In addition, the use of prickly pears as a source of betalains may be interesting since the plants of the *Opuntia* genus need minimal requirements from soil and water[2-10].

Despite their colouring capacity and superior antioxidant activity, betalains have not been considered by the food industry as potential additives. This is in part due to their instability, which prevents long-term storage[6]. Betalains stability is affected by temperature, pH, oxygen, exposure to light, aqueous activity and enzymatic activities; moreover, temperature is the most decisive factor for betalain decomposition[10-12].

The stabilization of betalains could be improved using microencapsulation technologies. Most of the studies are focused on the development of encapsulated hydrophilic forms using spray drying techniques. Recent studies have described preparation of spray-dried hydrophilic formulations containing betalains from red beet, cactus pear and amaranth [4, 6, 8, 10, 11, 13-15]. However, the use of these hydrophilic forms to colour food lipophilic matrices is very limited.

During the last years, Supercritical Fluid Technology, namely Particles from Gas Saturated Solutions (PGSS®) methodology has been used by several authors to incorporate bioactive compounds in lipophilic matrices. In this process, the compounds are melted and mixed with carbon dioxide in supercritical conditions (temperature>31 °C, pressure>7.4 MPa) forming a gas-saturated solution which is subsequently expanded to atmospheric conditions through an atomization nozzle. During the expansion, carbon dioxide is suddenly vaporized and intensely cooled down, thus providing the driving force for the solidification of the solute [16]. The PGSS® process is especially suited for processing polymers and lipids in which CO_2 has a large solubility and a melting depression effect [17]. For this application the plasticizing and swelling effect caused by CO_2 dissolution are particularly important for the improvement of the active substances incorporation. The high concentration of gas in the liquid phase leads to a considerable reduction in the melting point, viscosity and interfacial tension, helping to render substances sprayable which under classical conditions can hardly be sprayed or can even not be sprayed at all [18]. Within this context, PGSS® methodology seems to be an alternative to the conventional precipitation processes for the development of lipidic particles of betalains.

The purpose of this study is to exploit the use of Supercritical Fluid Technology, namely PGSS methodology, to develop lipophilic forms of a natural betalain-rich extract derived from *Opuntia* spp. juice by-products (fruit peels and seeds). Response Surface Methodology (RSM) was used to model the encapsulation of the natural pigment, namely betacyanins, into a lipophilic carrier (glyceryl monoostearate). This carrier was selected once it is food-grade, being a food additive approved in the EU (E471). Apart from that, it presents a suitable melting point for betacyanins encapsulation. The influence of process conditions on the antioxidant capacity, color and morphology of particles was also studied. Finally, the incorporation of the developed betalain lipophilic forms was evaluated in a dairy product (ice cream).

The novelty of this work is that an hydrophilic pigment is encapsulated in a lipophilic carrier to produce a lipophilic form, using a totally green procedure. To prepare liposoluble forms using conventional techniques, higher temperatures or organic solvents are involved, compromising the viability of the process and the quality and stability of the pigments. The technology we are presenting is totally GRAS without contact with volatile organic solvents and allowing the operation at inert atmosphere (absence of oxygen), avoiding oxidation reactions and operating at mild conditions concerning temperature.

Experimental

Materials

Reagents used for PGSS methodology were: Carbon dioxide (99.95% purity, Air Liquide, Lisbon, Portugal), Lumulse GMS K (Glyceryl monoostearate, HLB=3.9, Lambent Technology, Gurnee, USA, CAS n.o. 31566-31-1), Inwitor 600 (Polyglyceryl-3 Polyricinoleate, HLB=4, Sasol, Witten, Germany, CAS n.o. 68936-89-0).

Extraction experiments: Ethanol absolute 99,9% (Scharlau, Barcelona, Spain) and distillate water.

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 floride tetrahydrate (CoF_2), hydrogen peroxide (H_2O_2) and picolinic acid ($C_6H_5NO_2$) from Sigma- Aldrich (St Quentin Fallavier, France) and $FeCl_3$ from Riedel-de-Haën (Seelze, Germany). Disodium fluorescein (FL) was from TCI Europe (Antwerp, Belgium).

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).

Solvent extracts from *Opuntia* juice residues

Wild *Opuntia* spp. fruits were harvested by hand during October 2012. Fruits were collected from a plant growing in the South of Portugal (Algarve- Quarteira - N37º04.400, W008º06.100). All fruits were harvested at comparable ripening stages (physiological maturity).

Opuntia juice residues (peels and seeds) from fruits were submitted to a hydroalcoholic extraction. Residues were extracted in the dark with EtOH:H_2O (50:50 v/v) solution (ratio 1:20, w/v), for 2h at room temperature and 200 rpm (IKA® dual-speed mixer RW 20.n, Staufen, Germany). The extracts were centrifuged (9000rpm, 10min) and the supernatants were concentrated in a rotary evaporator at 40ºC under reduced pressure (58mbar). After the removal of the alcoholic fraction, the extracts were freeze dried at -20ºC (FreeZone Plus 4.5 L Cascade Freezer Dry System, LABCONCO®, Kansas City, United States of America), during 48h. The resulting extracts were kept in a cold, dry and dark environment until further analyses.

Emulsion preparation

Water-in-Oil (W/O) emulsion was prepared by melting previously the lipid (Glyceryl monoostearate), and adding the emulsifier (Polyglyceryl-3 Polyricinoleate). The lyophilized *Opuntia* spp. extract was solubilized in distilled water (20%). Then, this aqueous phase containing betacyanin extract and water was dispersed in the oil phase with a ratio extract:carrier of 1:3. The vial containing the previous materials was stirred for 5 minutes while immersed in a water bath at temperatures between 60-70°C.

Precipitation by Particles from Gas saturated Solutions (PGSS®)

Lipophilic forms of betalains were produced using the PGSS® technique. The RSM was used to model the encapsulation of betacyanins and optimize encapsulation conditions. The encapsulation of betacyanins through PGSS® were carried out following a Central Composite Rotatable Design (CCRD), as a function of three factors: pressure, temperature and equilibrium time (see electronic supplementary information). The schematic representation of the modified PGSS equipment (Separex Supercritical & High Pressure Technology) used to produce the particles is shown in Figure 1. Carbon dioxide was fed by a high-pressure piston pump (29723-71, Haskel International Inc., CA, USA) to a 50 cm^3 electrically thermostated high-pressure stirred vessel, containing the emulsion, until the desired working pressure was reached. After an equilibrium time at 150 rpm, the mixture was depressurised by an automated depressurisation valve and atomised through a two-fluid nozzle of 250 µm of diameter with external mixing (Spraying Systems Co., Air atomization 1/4J-SS, Separex, France) to a cyclone, where it was mixed with compressed air (0.7 MPa) for a better drying. Finally the particles were recovered in an 18 L collector vessel at atmospheric pressure.

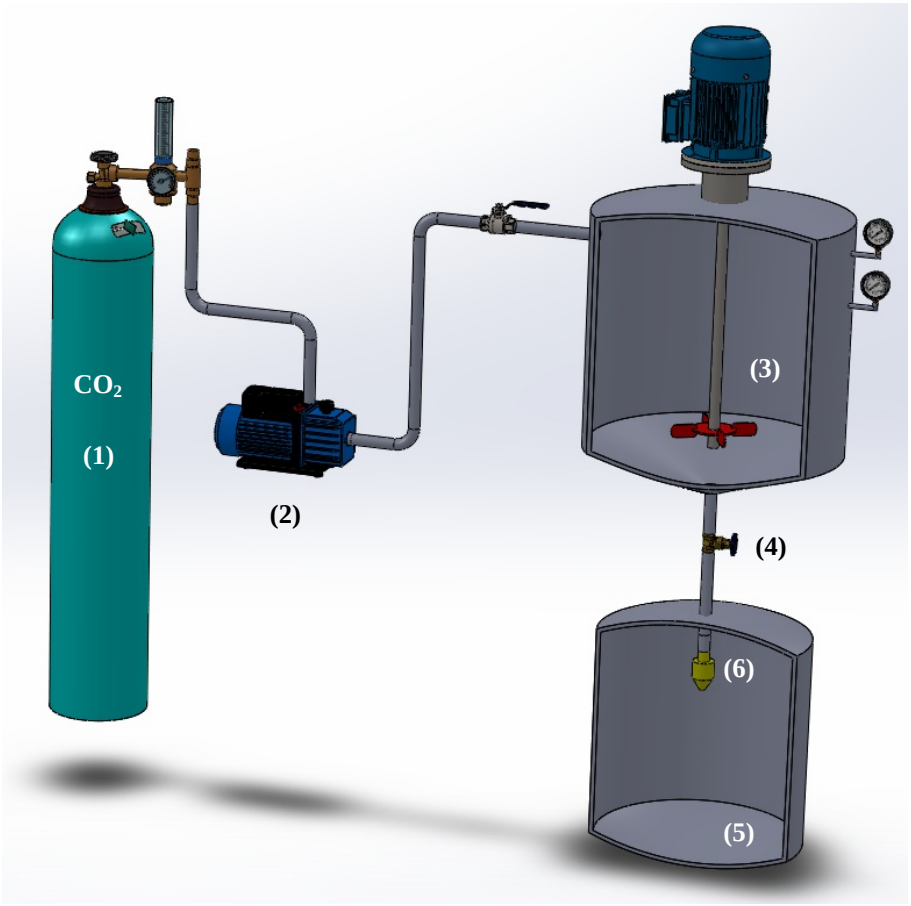


Figure 1. Experimental setup: (1) CO_2 cylinder (2) pneumatic piston pump (3) stirred vessel (electrically thermostated) (4) automated depressurisation valve (5) recovery vessel (6) nozzle.

Opuntia spp. extract and *Opuntia* spp. particle characterization

Scanning Electron Microscopy (SEM)

Particle morphology was observed by scanning electron microscopy (FEG-SEM) (Jeol, JSM-5310 model, Japan) at 20/25 kV, samples were coated with approximately 300 Å of gold in argon atmosphere.

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry measurement was carried out on a DSC TA instruments Q200 with module MDSC, to check the melting point of the particles and associated enthalpy. The samples were placed in an aluminium pan and sealed; the probes were heated from 20°C to 120°C at a rate of 1°C/min under nitrogen atmosphere. Measurements were done in triplicate. The particles (produced at the same time and process conditions) were also stored at the fridge (+4°C), during 2 months. DSC analysis was performed to samples in order to check state modifications.

Colour parameters (CIElab Method)

The colour of *Opuntia* dried extract and *Opuntia* particles 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 color 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 determine according to the expression $C^* = [(a^*) + (b^*)^2]^{1/2}$ and h° according to the expression $h^\circ = \arctan(b^*/a^*)$. These values were then converted to RGB (Red, Green, and Blue *color* values), using the software *OpenRGB* (Logicol).

Yield of Opuntia lipidic particles

The yield of the particles collected by PGSS was determined by weight measurements as the amount of the obtained particles in the sample collector divided by the amount of mass initially introduced in the mixing chamber.

Betacyanin content of extract and particles

The total betacyanin content of the *Opuntia spp.* extracts and particles was calculated according to Feliciano *et al.*, 2009 and Bolling *et al*., 2012[19, 20]. For the determination of the betacyanin content of the particles, it was important to ensure the total destruction of the particles and the total release of the betacyanins. Therefore, all the samples (particles) were previously dissolved in water (40 mg/mL) by ultrasonication extraction and filtered through a PVDF membrane (0.45 µm pore size). The extracts and particles were spectrophotometrically measured at 476, 538,

and 600 nm, as described by Guzman-Maldonado et al., 2010[21]. Betacyanins were determined with Nilsson equations:

$$\%\ Betacyanins = \frac{a}{1129} \times DF \times 100$$

where, $a = 1.095(A538 - A600), y = A476 - (A538 - a) - \left(\frac{a}{3.1}\right)$,

The results were presented as mg of betacyanin per 100 g of extract or particles (mg/100g) and were expressed as a mean of triplicates.

Antioxidant activity

For the antioxidant activity assays, the *Opuntia* lipidic particles were pre-treated according to the method described for the betacyanin content, concerning the total destruction of the particles.

Oxygen radical absorbance capacity (ORAC)

ORAC assay was carried out by the method of Huang *et al*., 2002[22] modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by Feliciano et al., 2009[20]. This assay measured the ability of the antioxidant species in the sample to inhibit the oxidation of disodium fluorescein (FL) catalyzed by peroxyl radicals generated from AAPH. Final ORAC values were calculated by EC (Effective Concentration) method to diminish the impact of the dilution effect as described by Bolling *et al*., 2012[19]. The results were presented as µmol of trolox equivalents antioxidant capacity (TEAC) per g of particles and were expressed as a mean of eight replicates.

Hydroxyl radical adverting capacity (HORAC)

The HORAC assay was based on a previously reported method[23] modified for the FL800 microplate fluorescence reader. This assay evaluates the hydroxyl radical prevention capacity of a sample using fluorescein as a probe. Final HORAC values were calculated by EC (Effective Concentration) method to diminish the impact of the dilution effect as described by Bolling *et al*., 2012[19]. Data was expressed as µmol of caffeic acid equivalents antioxidant capacity (CAEAC) per g of particles. 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[24] and adapted for the FLx800 fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA). 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. Final HOSC values were calculated by EmC (Effective Concentration) method to diminish the impact of the dilution effect

as described by Bolling *et al*., 2012[19]. Data was expressed as μmol of trolox equivalents antioxidant capacity (TEAC) per g of particles. Results were presented as a mean of eight replicates.

Additional details of these assays can be found at the electronic supplementary information.

Incorporation of Opuntia spp. extract and particles in a fat-based product

An ice-cream, produced by whipping slagroom and sugar, was used as a model of a fat-based product. The *Opuntia* spp. extract and the produced colorant powder were then added to the preparation, prior to the whipping in the concentration of 1.75mg/L. The mixtures were freeze and analysed visually, concerning the color homogenization.

Experimental design analysis / Statistical Analysis

The results of the CCRD, concerning the betacyanin content, antioxidant activity (ORAC, HORAC and HOSC), yield of collected particles and colour parameters were analysed using the software Statistica^{TM}, 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 by the determination coefficients (R^2) and adjusted R^2 (R_{adj}^2)[12, 25, 26].

Results and Discussion

Opuntia spp. extract characterization

The *Opuntia* spp. extract used to produce the lipidic particles were analysed in terms of betacyanin content and antioxidant activity (ORAC, HORAC and HOSC). The betacyanin content was found to be 332.9 mg/100g of dried extract and the ORAC, HORAC and HOSC values were 149.5±12.7, 74.9±7.2 and 119.0±14.4, respectively. Natural beet root juice used to produce microcapsules with gum arabic as a wall material have been found to have a betalain content of 13.5mg/100g[15]. Azeredo et al., 2007, conducted a study using maltodextrin to microencapsulate a beet root extract having a betacyanin content of 28.8mg/100g[13]. Hence, the produced extract is richer in betacyanins amongst other extracts from different sources in published work.

In Table 3, the colour parameters of the *Opuntia* dried extract and the commercial red beet pigment are presented. The colour parameters of the *Opuntia* dried extract were measured using CIELab method.

Table 1. Colour parameters of commercial red beet pigment and the produced powder (higher betacyanin content).

Variable, factors, unit	Colour parameters					Colour conversion to RGB
	L	a*	b*	h°	C*	
Opuntia dried extract	28.12	26.62	-2.22	355.70	26.71	
No.3600 (E162) [14]	51.54	19.64	0.71	2.00	n.i.	

n.i.: not indicated

Red beet pigment has been extensively commercialized as a food colorant. No. 3600 and E162 are the commercial codes for red beet pigment in U.S.A. and Europe, respectively The commercial red beet pigment presents a higher L* value than the *Opuntia* dried extract (L*=51.54), presenting a lighter colour. The a* value of the *Opuntia* dried extract was higher than the red beet pigment (26.62 and 19.64, respectively), indicating the red colour. The b* value of red beet pigment is 0.71 and the Opuntia dried extract is -2.22. This means that the red beet pigment has a more yellow colour and the dried extract a bluer colour. The red beet pigment has a lower h° (2.00), very similar to red colour. The h° value of the *Opuntia* dried extract is 355.70, indicating a slightly purple shade of red. This results, suggested that the produced *Opuntia* dried extract is very similar to the commercial red beet powder.

Modelling of betacyanins encapsulation through PGSS®

The encapsulation experiments were carried out according to the CCRD previously described (Table 1 and 2). The obtained results of the experiments, i.e. the betacyanin contents, antioxidant activity (ORAC, HORAC and HOSC), colour parameters and the yield of the collected particles, are shown in Table 4. These results were used to estimate both linear and quadratic effects of the variables and also their linear interactions (see supplementary information).

Table 2 Summary of experimental results.

Experiment number	Yield of collected particles (%)	Betacyanin content (mg/100g)	*ORAC* (μmol CAET/g part. or g dried ext.)	*HORAC* (μmol CAEAC/g part. or g dried ext.)	*HOSC* (μmol CAET/g part. or g dried ext.)	*Colour parameters* L*	a*	b*	h°	C*	RGB colour conversion
Opuntia spp. dried extract	-	332.9 ± 0.1	149.5 ± 12.7	74.9 ± 7.2	119.0 ± 14.4	28.12 ± 0.28	26.62 ± 0.52	-2.22 ± 0.07	355.70 ± 0.00	26.71 ± 0.52	
1	29	18.4 ± 0.0	10.70 ± 1.16	5.96 ± 0.95	9.00 ±1.27	85.94 ± 1.85	12.52 ± 0.83	-4.34 ± 0.35	340.88 ± 0.02	13.25 ± 0.87	
2	19	35.7 ± 0.1	17.45 ± 1.40	8.69 ± 1.02	13.08 ± 2.77	78.84 ± 1.24	19.37 ± 1.14	-6.51 ± 0.49	341.42 ± 0.01	20.43 ± 1.22	
3	17	19.9 ± 0.1	10.60 ± 0.65	4.04 ± 1.59	6.13 ± 0.80	89.87 ± 1.22	13.45 ± 0.27	-4.64 ± 0.20	342.89 ± 0.03	30.60 ± 0.26	
4	22	7.5 ± 0.3	8.31 ± 0.66	3.26 ± 1.14	4.55 ± 0.49	90.41 ± 0.74	7.35 ± 0.26	-1.51 ± 0.15	348.39 ± 0.02	7.50 ± 0.27	
5	12	21.4 ± 0.6	13.34 ± 1.36	6.90 ± 1.04	7.86 ± 0.28	83.92 ± 0.67	14.38 ± 0.79	-4.93 ± 0.40	341.08 ± 0.01	15.20 ± 0.87	
6	20	41.3 ± 0.5	21.87 ± 2.36	12.38 ± 1.13	19.56 ± 0.79	76.75 ± 0.29	21.25 ± 0.16	-6.76 ± 0.05	342.35 ± 0.00	22.30 ± 0.17	
7	31	26 ± 0.9	16.04 ± 2.07	6.70 ± 1.18	11.69 ± 0.79	84.54 ± 1.5	15.50 ± 0.63	-5.82 ± 0.22	339.42 ± 0.00	16.56 ± 0.67	
8	30	25.6 ± 0.9	15.65 ± 1.73	7.44 ± 1.07	12.74 ± 0.88	83.83 ±0.19	16.17 ± 0.21	-5.49 ± 0.09	341.25 ± 0.00	17.08 ± 0.22	
9	27	8.0 ± 0.1	7.06 ± 0.67	3.04 ± 1.06	5.66 ± 0.75	91.33 ± 0.22	8.65 ± 0.17	-2.71 ± 0.04	342.60 ± 0.00	9.06 ± 0.17	
10	21	27.2 ± 0.2	17.75 ± 1.69	5.68 ± 0.72	12.66 ± 1.22	85.30 ± 0.18	15.41 ± 0.28	-6.17 ± 0.09	338.18 ± 0.00	16.60 ± 0.29	
11	18	16.3 ± 0.9	10.82 ± 0.90	6.06 ± 0.93	8.36 ± 1.01	88.69 ± 0.11	11.15 ± 0.10	-3.59 ± 0.06	342.15 ± 0.00	11.71 ± 0.12	
12	22	16.8 ± 0.1	11.15 ± 1.08	3.25 ± 0.65	9.17 ± 1.07	87.75 ± 0.22	10.58 ± 0.23	-3.17 ± 0.07	343.32 ± 0.00	11.04 ± 0.24	
13	34	64.4 ± 4.5	24.01 ± 2.13	11.61 ± 1.05	18.33 ± 2.29	80.56 ± 0.93	19.39 ± 0.25	-7.21 ± 0.12	339.60 ± 0.01	20.69 ± 0.25	
14	23	17.3 ± 0.3	10.13 ± 0.90	4.34 ± 0.87	9.32 ± 1.37	87.11 ± 1.17	11.04 ± 1.01	-3.63 ± 0.46	341.80 ± 0.01	11.62 ± 1.10	
15	33	44.9 ± 0.6	22.17 ± 2.22	12.38 ± 0.90	18.25 ± 2.44	84.75 ± 0.10	14.27 ± 0.32	-4.40 ± 0.09	342.86 ± 0.00	14.93 ± 0.34	
16	21	43.2 ± 0.0	21.61 ± 2.30	5.26 ± 0.87	19.43 ± 2.69	82.37 ± 0.42	16.73 ± 0.59	-5.93 ± 0.19	340.48 ± 0.00	17.75 ± 0.62	
17	27	12.7 ± 3.3	9.93 ± 1.02	6.11 ± 0.87	10.01 ± 1.69	90.10 ± 0.36	8.98 ± 0.43	-2.51 ± 0.10	344.38 ± 0.00	9.2 ± 0.44	

The response surfaces fitted to betacyanin content (Figure 2), ORAC, HOSC and L^* value (see supplementary information) can be described by second-order polynomial models as a function of pressure, temperature and equilibrium time (Table 5).

Table 3. Model equations for the response surfaces fitted to the values betacyanins, ORAC, HOSC and L*, as a function of Pressure (P), Temperature (T) and equilibrium time (t), and respective R^2 and R_{adj}^2

POLYNOMIAL MODEL EQUATIONS	R^2	R_{adj}^2
$Betacyanins = 102.53 + 2.581P + 0.236P^2 + 4.59T + 0.02t^2 - 0.249PT + 0.119Pt - 0.048Tt$	0.715	0.493
$ORAC = -9.90 + 0.545P + 0.083P^2 + 1.294T + 0.006t^2 - 0.084PT + 0.049Pt - 0.017Tt$	0.748	0.553
$HOSC = 65.75 - 3.209P + 0.076P^2 - 0.361T + 0.0001t^2 + 0.0004Pt$	0.538	0.327
$L^* = 99.66 + 2.22P - 0.05P^2 + 0.514T + 0.0005Pt$	0.665	0.554

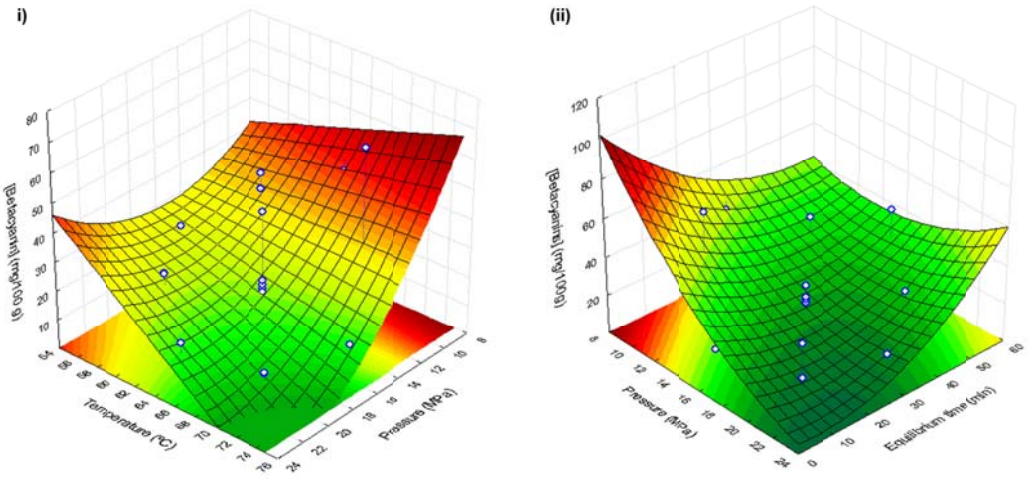


Figure 2. Response surface fitted to the betacyanin content as a function of (i) temperature and pressure and (ii) of equilibrium time and pressure.

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. It is better to accept a factor with a p value higher than 0.05 rather than to take the chance of missing an important factor[25]. The values for both R^2 and R_{adj}^2 of these models (Table 5) suggest a good agreement between the experimental data and the theoretical values predicted by the model. About 72% or 75 % of the observed results concerning betacyanins content and ORAC are explained by the respective models. However, no optimum conditions were observed in the response surface for the

betacyanin encapsulation. Therefore, only the identification of the region of the experimental domain corresponding to the best response can be achieved.

Analysing Figure 2, the P had a negative effect of the betacyanin encapsulation. Thus, lower pressures lead to an increase in the betacyanin encapsulation. This effect was more pronounced at higher temperatures and lower equilibrium time. At these conditions, the particles presented a pigment content of 64.4 ± 4.5 mg/100g. This result may be explained by the negative impact of higher pressure on the emulsion stability. Higher temperatures also help in the homogenization of the mixture, as well as lower equilibrium times are preferred probably due to the low shelf-life stability of the emulsion.

Some previous studies were conducted by other authors on the encapsulation of pigments from *Opuntia* spp. Saenz *et al*., 2009 encapsulated a betalain-rich extract from *Opuntia ficus-indica* using maltodextrin and inulin through spray-drying technique. The maximum content of the encapsulated pigment was 60.0 and 64.0 mg of betacyanin per 100g of powder for maltodextrin and inulin, respectively[11]. Another study of the encapsulation of a betacyanin-rich extract from *Opuntia lasiacantha* was performed using maltodextrin through spray-drying [27]. The amount of betacyanin in powder after drying was 65.7 mg of betanin per 100g. The maximum content of the encapsulated pigment in this work was 64.4 ± 4.5 mg/100g, being similar with other published work using other processes and carriers[11, 27].

The repeatability (coefficient of variation) of the encapsulation process through PGSS® was around 5 %, taking into account three samples of the design (centre points).

The antioxidant activities obtained were between 7.06 and 24.01 μmol CAET/g of particles for ORAC, 3.04 and 12.38 μmol CAEAC/g of particles for HORAC, 4.55 and 19.43 μmol CAET/g of particles for HOSC (Table 3). From the results of HOSC, ORAC and HORAC and it is possible to visualize that as the betacyanin content of the samples increase, the antioxidant activity also increases. There is a linear correlation between the betacyanin content and the antioxidant values with a correlation factor (R^2) of 0.84, 0.91 and 0.67, for HOSC, ORAC and HORAC, respectively (Figure 3).

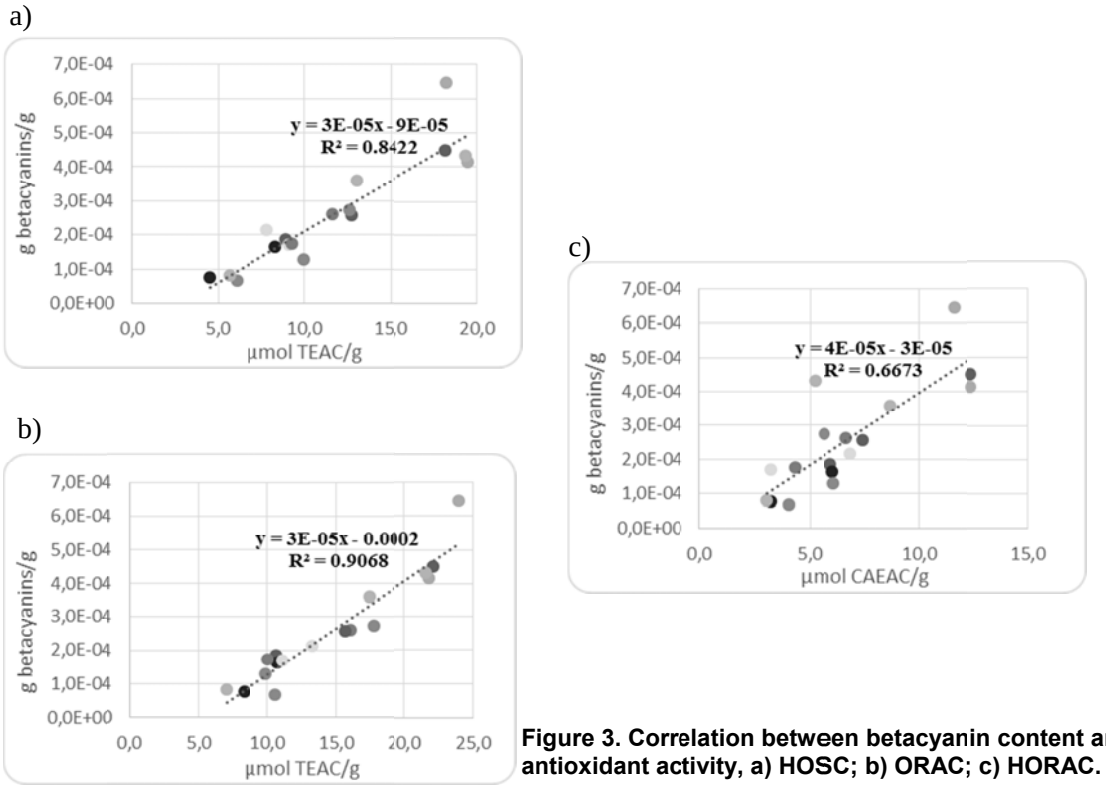


Figure 3. Correlation between betacyanin content and antioxidant activity, a) HOSC; b) ORAC; c) HORAC.

As shown in Table 3, the yield of collected particles (mass of particles collected/mass of product introduced in the pressure vessel) was low in all experiments (from 12 to 34%), indicating a loss of product and possibly of fine particles. De Paz, et. al, 2012, has conducted a study of formulation of beta-carotene using the same precipitation process (PGSS®) and has obtained a similar process yield (5-44%)[16].

Particle morphology by Scanning Electron Microscopy

SEM Microphotographs of the powders produced by PGSS process for comparable conditions are shown in Figure 4. When processing at lower temperatures (60°C), a greater degree of shrinkage is observed than when processing at higher temperatures (70°C). Mechanisms involved in shrinkage and deformation are more pronounced when processing at low temperatures since water diffusion is slower, allowing more time for structures to deform, shrink, and collapse. These results are in line with what has been obtained for indicaxanthin encapsulation in maltodextrin using spray drying [4]. The average size of particles was 10 µm, approximately. Furthermore, as pressure is increased, larger amounts of CO_2 are dissolved in the melted carrier and higher pressure drop is produced across the nozzle; therefore, more CO_2 gas bubbles are formed increasing the cooling rate which originates porous particles as the gas cannot diffuse out of the particles perforating particle surface. On the contrary, as temperature is higher, the solubility of CO_2 is decreased, allowing the formation of more spherical structures since the slower solidification of the droplets facilitates the diffusion of CO_2 out of the particles[17,

[28]. Regarding the betacyanin content there is no correlation between the particle morphology and the incorporation of the pigment, as can be seen in Figure 4.

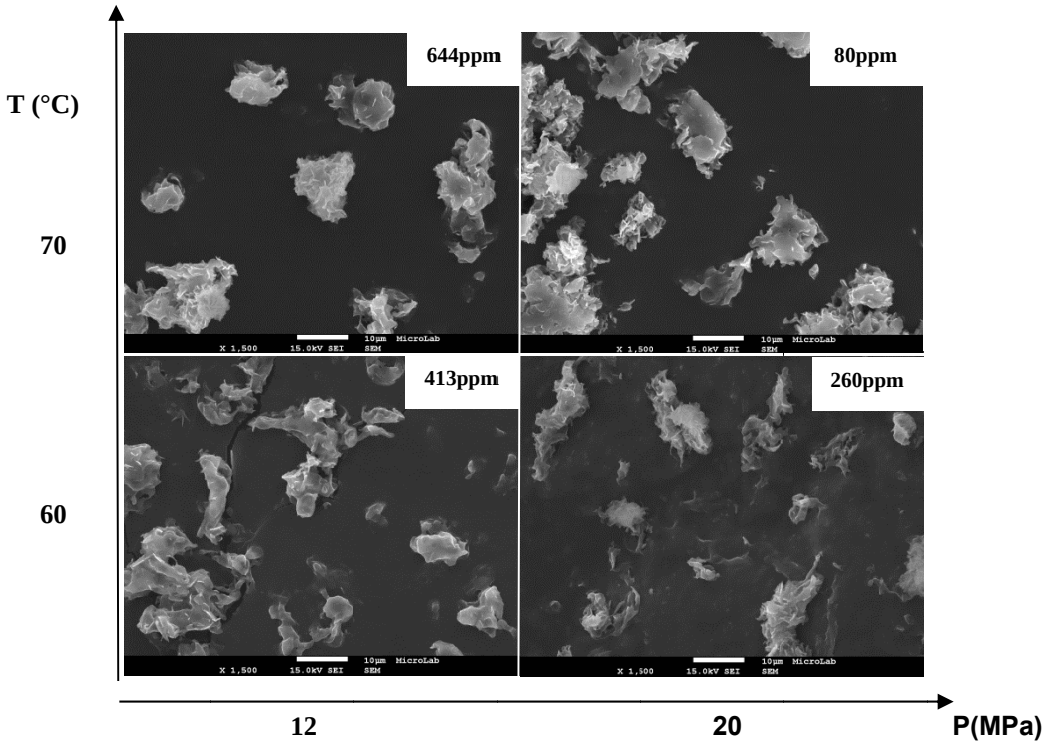


Figure 4. SEM pictures at different conditions of pressure and temperature and an equilibrium time of 15 minutes and corresponding betacyanin contents (ppm)

Determination of the melting point for the particles with higher betacyanin content

The melting point was evaluated for the particles with higher betacyanin content. The thermogram is presented in Figure 5.

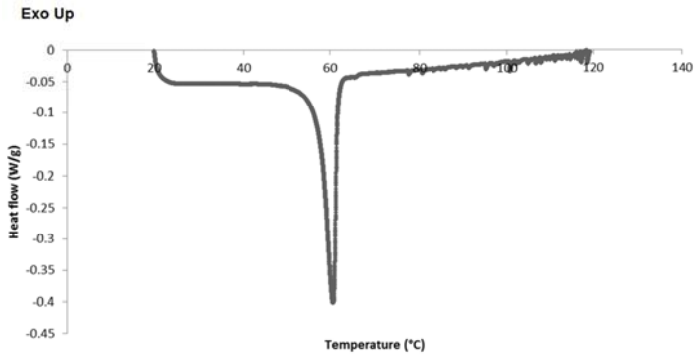


Figure 5. Thermogram of particles with highest betacyanin content

From the thermogram presented above, the melting temperature of the particles having the highest betacyanin content was between 57 and 65°C (Figure 5).

Figure 6 resumes the results of enthalpy for the optimized conditions after 2 months of storage at ambient temperature and at fridge conditions (4˚C).

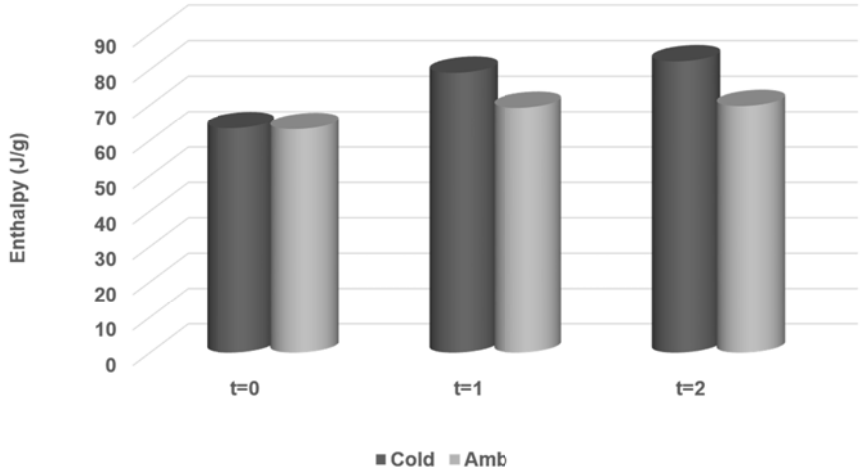


Figure 6. Enthalpy differences between particles stored at ambient temperature and cold conditions at different times of storage.

Analysing the results, it is possible to visualize an increase of enthalpy values throughout time for samples stored at ambient temperature and at 4˚C. According to Bunges & Unruh, 2007, a gradual increase in melting enthalpy over, e.g., several days or weeks of storage is not uncommon in dispersions of solid lipid nanoparticles as a result of polymorphic transitions and the formation of a higher degree of crystalline order [29]. When comparing the enthalpy of samples stored at cold conditions and ambient temperature, it is possible to visualize higher enthalpy values at cold conditions. This means that at these conditions, a crystalline state is more pronounced and this can have an impact on the betacyanin content throughout the time.

Colour parameters

The colour of the *Opuntia* particles was measured using CIELab method. The results are presented in Table 3.

From the results in Table 3, it is possible to conclude that the colour parameters do not translate the betacyanin content that is incorporated in the particle. This means that the pigment can be more encapsulated (in the interior) rather than at the particle surface. An example of this is sample 13 that presented the highest content in betacyanins but did not present the lowest L* value (80.56). Regarding the C* and h° values, among all samples of the design, the h° value is between 338.28 and 348.39 and the C* value between 7.50 and 30.60, indicating a slightly purple shade of red.

Incorporation in a fat-based product

The *Opuntia spp*. extract and the particles were added to the ice cream formulation in a concentration of 1.75ppm. Pictures have been taken after freezing and the images are shown in Figure 7.

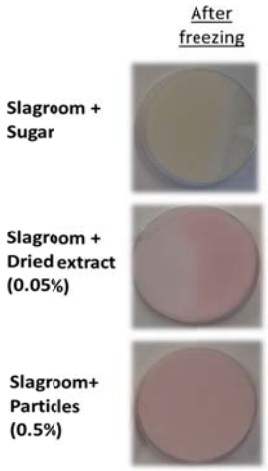


Figure 7. Incorporation of particles and dried extract in a food product (ice-cream)

From the images it is possible to see a good homogenization of the pink colour of the product after the incorporation of lipophilic particles, in contrast to the dried extract that caused colour separation in the ice-cream.

Conclusions

In this work, betacyanin pigments derived from *Opuntia* juice by-products were successfully encapsulated in a lipophilic carrier through supercritical fluid technology, namely PGSS. By using the statistical tool of Response Surface Methodology, it was possible to model the pigment encapsulation and optimize encapsulation conditions. The highest amounts of encapsulated betacyanin were achieved at lower pressures. This effect was more pronounced at higher temperatures and lower equilibrium times (12MPa, 70^{o}C and 15min). Under these conditions, *Opuntia* spp. particles presented 64.4 mg/100g of betacyanins and high antioxidant capacity, allowing a good pink colour homogenization after incorporation in a fat-based food product. From the results obtained it can be concluded that PGSS can be considered as a promising technology to develop lipophilic forms of a natural red/pink natural colorant for the application in food industry.

Acknowledgements

The authors acknowledge the financial support received from Portuguese Fundação para a Ciência e Tecnologia (FCT) through PTDC/AGR-AAM/099645/2008 project, PEst-OE/EQB/LA0004/2011 grant and for the doctoral (SFRH / BDE / 51856 / 2012) fellowship.

The authors are grateful to Eng Armando Ferreira for the use of Minolta Colorimeter CR-200 at INIAV (Instituto Nacional de Investigação Agrária e Veterinária).

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Electronic Supplementary Information

EXPERIMENTAL

Experimental design (Process Optimization)

RSM was used to model the encapsulation of betacyanins and optimize encapsulation conditions. The encapsulation of betacyanins through PGSS® were carried out following a Central Composite Rotatable Design (CCRD), as a function of three factors: pressure, temperature and equilibrium time. A total of 17 experiments were performed: 8 factorial points (coded levels as (+1) and (-1); 6 star points (coded as (+α) and (-α)); 3 centre points (coded as 0) (Table 1).

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

Variable, factors, unit	Levels				
	-α.	-1	0	+1	+α
Pressure, X_1 (MPa)	9	12	16	20	23
Temperature, X_2 (°C)	57	60	65	70	73
Equilibrium time, X_3 (min)	5	15	30	45	55

The pressure varied from 9 to 23 MPa, the temperature from 57 to 73^{0}C and the equilibrium time from 5 to 55min, according to the experimental design followed (Table 2). A total of 17 assays including three replicates of the centre points were generated. The repetitions of the centre points are used to determine the experimental error, which is assumed to be constant along the experimental domains. Experiments were conducted randomly, according to the methodology described in PGSS® technique.

Table 2. The central composite design for the three independent variables

Experiment number	Pressure, X_1 (MPa)	Temperature, X_2 (°C)	Equilibrium time, X_3 (min)
1	16 (0)	65 (0)	30 (0)
2	12 (-1)	60 (-1)	45 (+1)
3	16 (0)	65 (0)	30 (0)
4	20 (+1)	70 (+1)	45 (+1)
5	16 (0)	65 (0)	30 (0)
6	12 (-1)	60 (-1)	15 (-1)
7	20 (+1)	60 (-1)	15 (-1)
8	20 (+1)	60 (-1)	45 (+1)
9	20 (+1)	70 (+1)	15 (-1)
10	16 (0)	57 (-1.68)	30 (0)
11	12 (-1)	70 (+1)	45 (+1)
12	23 (+1.68)	65 (0)	30 (0)
13	12 (-1)	70 (+1)	15 (-1)
14	16 (0)	65 (0)	5 (-1.68)
15	16 (0)	65 (0)	55 (+1.68)
16	9 (-1.68)	65 (0)	30 (0)
17	16 (0)	73 (+1.68)	30 (0)

Oxygen radical absorbance capacity (ORAC)

ORAC assay was carried out by the method of Huang *et al*., 2002[22] modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by Feliciano et al., 2009[20]. This assay measured the ability of the antioxidant species in the sample to inhibit the oxidation of disodium fluorescein (FL) catalyzed by peroxyl radicals generated from AAPH. Briefly, 25 µL of the appropriate sample dilutions and 150 µL of disodium fluorescein (2×10^{-7} mM) were added to a 96-well microplate. The microplate was put in a fluorescent reader and allowed to incubat at 37 ºC, for 10 minutes. The reaction was started with 25 µL of AAPH (153 mM) added through the injector. Fluorescence emitted by the reduced form of FL was measured in an FL800 microplate fluorescent reader (Bio-Tek Instruments, Winooski, VT, USA) and recorded every 1 minute at the emission wavelength of 530 ± 25 nm and excitation wavelength of 485 ± 20 nm for a period of 40 minutes. Phosphate buffer (75 mM, pH=7.4) was used to prepare AAPH and FL solutions and as blank. Solutions of 5, 10, 20, 30, and 40 µmol/L of Trolox were used as control standards. Final ORAC values were calculated by EC (Effective Concentration) method to diminish the impact of the dilution effect as described by Bolling *et al*., 2012[19]. The results were presented as µmol of trolox equivalents antioxidant capacity (TEAC) per g of particles and were expressed as a mean of eight replicates.

Hydroxyl radical adverting capacity (HORAC)

The HORAC assay was based on a previously reported method[23] modified for the FL800 microplate fluorescence reader. This assay evaluates the hydroxyl radical prevention capacity of a sample using fluorescein as a probe.

Briefly, 30 µL of appropriate sample dilutions and 170 µL of FL ($9.28\text{x}10^{-8}$ M) were added to a black 96-well microplate. Then, 40 µL of hydrogen peroxide (H_2O_2), 0.206 M, were added to each well of the microplate. Finally, the reaction was started by adding 60 µL of cobalt (II) fluoride (CoF_2), 1.15 mM, to the mixture previously placed in the microplate. Sodium phosphate buffer (SPB), 75 mM, pH=7.4, was used to prepare the solution of FL, H_2O_2 and CoF_2 were prepared with Milli-Q water. Caffeic acid was used as a standard, and 50, 100, 150, 200 and 250 µM solutions in acetone:Milli-Q water (50:50, v/v) were used to create the calibration curve. Acetone:Milli-Q water (50:50, v/v) solution was used to prepare the samples and as a blank. The fluorescence emitted by the reduced form of FL was measured and recorded every 1 minute during 60 minutes, at 37 ºC. The FLx800 fluorescence microplate reader was controlled by software Gen5 and was used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. Final HORAC values were calculated by EC (Effective Concentration) method to diminish the impact of the dilution effect as described by Bolling *et al*., 2012[19]. Data was expressed as µmol of caffeic acid equivalents antioxidant capacity (CAEAC) per g of particles. 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[24] and adapted for the FLx800 fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA). 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.

Briefly, 30 µL of appropriate sample dilutions, 40 µL of H_2O_2 (0.1990 M) and 170 µL of FL (9.28x10-8 M) were added to a black 96-well microplate. The reaction was started by adding 60 µL of iron (III) chloride ($FeCl_3$), 3.43 mM, to the wells of the microplate. SPB, 75 mM, pH=7.4, was used to prepare the solution of FL, and the solutions of H_2O_2 and $FeCl_3$ were prepared with Milli-Q water. Trolox was used as a standard, and 5, 10, 15, 20 and 30 µM solutions in acetone:Milli-Q water (50:50, v/v) were used to perform the calibration curve. Acetone:Milli-Q water (50:50, v/v) solution was used to prepare the samples and as a blank. The fluorescence emitted by the reduced form of FL was measured and recorded every 1 minute, during 60 minutes, at 37 ºC. The FLx800 fluorescence microplate reader was controlled by software Gen5 and was used with fluorescence filters for an excitation wavelength of 485±20 nm and an emission wavelength of 530±25 nm. Final HOSC values were calculated by EC (Effective Concentration) method to diminish the impact of the dilution effect as described by Bolling *et al*., 2012[19]. Data was expressed as µmol of trolox equivalents antioxidant capacity (TEAC) per g of particles. Results were presented as a mean of eight replicates.

RESULTS AND DISCUSSION

The effects of each factor and the interactions between factors on the various responses were calculated. Table 1 shows the linear and quadratic effects of each variable and of their interactions on the betacyanin content, ORAC, HOSC and L* value during the encapsulation process. For HORAC and yield of collected particles, a lack of fit of the polynomial models exhibited by low values of R^2 andR_{adj}^2 was observed.

Table 3. Linear (L) and quadratic (Q) effects and respective significance levels (p) of the tested variables [factors: Pressure (P), Temperature (T) and Equilibrium time (t)] and interactions on betacyanin content, ORAC, HOSC and L*

Factor	Betacyanins		ORAC		HOSC		L*	
	Effect	p value	Effect	p value	Effect	p value	Effect	p value
P (L)	-19.81	0.019[a]	-5.97	0.032[a]	-6.14	0.005[a]	5.04	0.019[a]
P (Q)	7.48	0.335[b]	2.99	0.266[b]	3.41	0.018[a]	-1.91	0.330[b]
T (L)	-8.32	0.243[b]	-5.58	0.041[a]	-3.61	0.165[b]	5.14	0.017[a]
T (Q)	0.31	0.967	1.17	0.649	1.30	0.112	-0.02	0.991
t (L)	-1.20	0.860	0.67	0.775	-0.20	0.570	0.68	0.694
t (Q)	8.20	0.291[b]	2.79	0.294[b]	3.03	0.923[b]	-1.27	0.505
P x T	-9.95	0.282[b]	-3.35	0.289[b]	-2.03	0.207	-0.07	0.974
P x t	13.20	0.166[b]	5.80	0.088[b]	4.08	0.460[b]	-2.96	0.211[b]
T x t	-10.65	0.252[b]	-3.00	0.339[b]	-1.38	0.160	1.46	0.520

[a] Significant effects with $p \leq 0.05$.
[b] Effects with $p > 0.05$ considered in the model.

For the results obtained for betacyanins, a negative significant effect of P and T on betacyanin encapsulation indicated that higher P and T values, within the tested range, correspond to a lower encapsulation of betacyanins. This result can be explained by the emulsion instability at higher pressures or the pigment degradation under these conditions. The t has demonstrated to have lower effect on the encapsulation of betacyanins. The positive quadratic effect of t indicated that the experimental results on betacyanin encapsulation can be fitted to a four-dimensional concave surface. All the interactions between factors for the betacyanin content were important. As the P and T values increased, the betacyanin encapsulation decreased. When the P and t increased the betacyanin encapsulation increased. As T and t increased, the encapsulation of betacyanins decreased.

Concerning the ORAC values, significant linear negative effects of P and T were found, in contrast to the observations for the betacyanin content. As P and T increased, the ORAC values decreased. Also, the significant quadratic positive effects of P and t indicated that ORAC can be described by a four dimensional concave surface. When the P and t increased the ORAC increased and as T and t increased, the ORAC values decreased.

Regarding the HOSC values, significant linear negative effects of P and T were found, in accordance with the observations for the ORAC values. As P and T are increased the HOSC

values decreased. Also, the significant quadratic positive effects of P and t indicated that ORAC can be described by a four dimensional concave surface. When the P and t increased, the HOSC values increased, as well.

Finally, for the results of L^*, the same trend was verified as with the ORAC and HOSC values. As P and T are increased, the L^* values increased (lighter particles are obtained). Also, the significant quadratic positive effects of P and t indicates that the L^* value can be described by a four dimensional convex surface. When the P and t increased, the L^* values decreased.

The response surfaces fitted to ORAC, HOSC and L^* value are presented below (Figure 1).

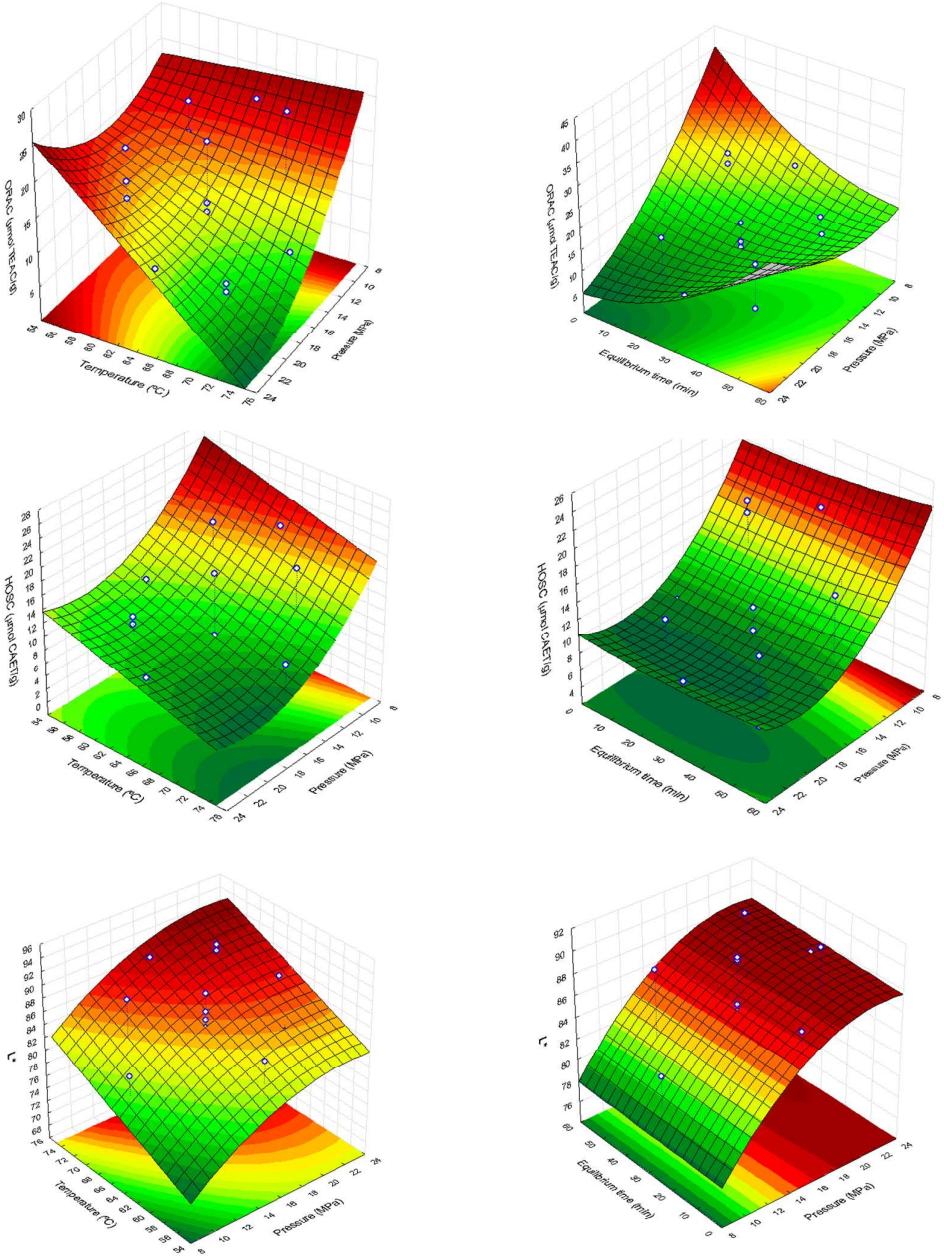

Figure 1. Response surfaces fitted to the ORAC, HOSC and L* value as a function of (i) temperature and pressure and (ii) of equilibrium time and pressure.