Ana Nunes Nunes

Degree in Biochemistry

Isolation and encapsulation of a natural colourant using Green Technologies

Dissertation to obtain master degree in
Food Technology and Safety

Supervisor: Catarina Duarte, Ph.D, IBET/ITQB-UNL
Co-supervisor: Ana Lúcia Leitão, Ph.D, Assistant Professor FCT-UNL

Jury:

President: Doctor Benilde Simões Mendes
Arguer: Doctor Ana Vital Morgado Marques Nunes
Supervisor: Doctor Catarina Maria Martins Duarte

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Acknowledgments

Agradeço à minha orientadora, a Dra. Catarina Duarte, por me ter encorajado e motivado a iniciar o mestrado, pelas condições que me proporcionou para desenvolver o meu trabalho bem como pela atenção e apoio que prestou ao longo da sua orientação.

Agradeço à minha co-orientadora, a Prof. Dra. Ana Lúcia Leitão, por todos os conhecimentos transmitidos, toda a disponibilidade, apoio e preocupação que prestou ao longo destes dois anos.

Estou grata a toda a equipa dos Nutracêuticos e Libertação Controlada, Teresa Serra, Ana Matias, Joana Poejo, Sara Nunes, Agostinho Alexandre, Daniel Deodato, Vanessa Gonçalves, Cátia Carmo, Inês Silva, Liliana Rodrigues, Catarina Maia e Arturo Álvarez-Bautista, por me terem proporcionado um ambiente agradável no meu dia-a-dia e por todos os conhecimentos e ideias partilhados.

Agradeço também de forma especial à Cátia, estou muito grata pela enorme dedicação ao meu trabalho, pelas inúmeras discussões científicas, pelo apoio e motivação nos momentos mais difíceis.

Agradeço ao Engenheiro Armando Ferreira do Instituto Nacional de Investigação Agrária e Veterinária por disponibilizar o aparelho Minolta Colorimeter CR-200.

Agradeço à Engenheira Isabel Nogueira do laboratório ICEMS do Instituto Superior Técnico pela disponibilidade e apoio nas análises de SEM.

Agradeço à minha família e amigos, especialmente à Alice, Antónia, Amália e Bárbara, pelo constante interesse em entender o meu trabalho, pelo apoio incondicional e pela força e motivação nos momentos mais difíceis.

Agradeço de uma forma muito especial ao Marcelo, por toda a força, apoio e admiração ao longo destes anos.

Abstract

The use of natural pigments instead of synthetic colourants is receiving growing interest in the food industry. In this field, cactus pears (*Opuntia* spp.) have been identified to be a promising betalainic crops covering a wide coloured spectrum.

The aim of this work was to develop adequate clean and mild methodologies for the isolation and encapsulation of betacyanins, from cactus pear fruits (*Opuntia* spp.).

Firstly, two different emerging technologies, namely PLE (Pressurized Liquid Extraction) and HPCDAE (High Pressure Carbon Dioxide-Assisted Extraction), were exploited to isolation of betacyanins form cactus pear fruits. Different process conditions were tested for the maximum recovery of betacyanins. Results showed that highest extraction yields were achieved for HPCDAE and mass ratio of pressurized carbon dioxide *vs*. acidified water was the parameter that most affected the betacyanins extraction. At optimum conditions of HPCDAE, *Opuntia* spp. extract presented a total betacyanin content of 211 ± 10 mg/100 g whereas extracts obtained using conventional extraction, PLE in static and in dynamic mode presented a total betacyanin content of 85 ± 3, 191 ± 2 and 153 ± 5 mg/100 g, respectively. HPCDAE has proven to be a successful technology to extract betacyanins from *Opuntia* spp. fruits.

Afterward, Supercritical Fluid Technology was exploited to develop lipidic particles of betalain-rich extract. A betacyanin-rich conventional extract was encapsulated by PGSS® (Particles from Gas Saturated Solutions) technique. Different process conditions were tested in order to model the encapsulation of betacyanins. The pressure had a negative effect on betacyanin encapsulation. Lower pressures leads to an increase in the betacyanin encapsulation. This effect was more pronounced at higher temperatures and lower equilibrium time. At these conditions, *Opuntia* spp. particles presented 64.4 ± 4.5 mg/100 g and high antioxidant capacity. When compared with the *Opuntia* spp. dried extract, lipidic particles contributed to a better homogenization of the pink colour after incorporation in ice cream.

Keywords: natural colour; betacyanins; *Opuntia* spp.; HPCDAE; PLE; PGSS®.

Resumo

O uso de pigmentos naturais em alternativa aos corantes sintéticos tem apresentado um crescente interesse na indústria alimentar. O género *Opuntia* destaca-se como uma matéria-prima promissora rica em betalaínas, abrangendo um amplo espectro de cor.

O principal objetivo deste trabalho consistiu no desenvolvimento de metodologias limpas para o isolamento e aumento de estabilidade de betacianinas (betalaínas) de frutos *Opuntia* spp.

Neste sentido, duas tecnologias diferentes, nomeadamente PLE (extração com fluidos pressurizados) e HPCDAE (extração assistida com dióxido de carbono pressurizado), foram exploradas para isolar betacianinas de frutos *Opuntia* spp. Foram testadas diferentes condições de processo com o intuito de maximizar o teor de betacianinas. Os resultados demonstram que o rendimento de extração de betacianinas mais elevado foi obtido pela HPLCAE e que a razão mássica entre o dióxido de carbono pressurizado e a água acidificada foi o parâmetro que mais afetou a extração deste pigmento. Nas condições ótimas da HPCDAE, o extrato de *Opuntia* spp. obtido apresenta um teor de betacianinas totais de 211 ± 10 mg/100 g, enquanto que os extratos obtidos através da extração convencional, PLE no modo estático e no modo dinâmico, apresentam teores de betacianinas totais de 85 ± 3, 191 ± 2 e 153 ± 5 mg/100 g, respetivamente. A HPCDAE provou ser uma tecnologia de sucesso na extração de betacianinas dos frutos *Opuntia* spp.

Por outro lado, a tecnologia de fluidos supercríticos foi explorada para aumentar a estabilidade das betacianinas. Para tal, um extrato convencional rico em betacianinas foi encapsulado, utilizando o PGSS®. Foram testadas diferentes condições de processo com o objetivo de maximizar o teor de betacianinas encapsulado. A pressão apresentou um efeito negativo na encapsulação das betacianinas. Baixas pressões conduzem ao aumento na encapsulação do pigmento. Nestas condições, as partículas de *Opuntia* spp. apresentam um teor de betacianinas totais de 64.4 ± 4.5 mg/100 g e uma elevada atividade antioxidante. Quando comparadas com o extrato seco de *Opuntia* spp., as partículas lípicas contribuíram para uma melhor homogeneização da cor rosa após sua incorporação num gelado.

Termos chave: cores naturais; batacianinas; *Opuntia* spp.; HPCDAE; PLE; PGSS®.

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List of abbreviations, acronyms and symbols

Abbreviation	Full form
APPH	2`,2`-Azobis (2-amidinopropane) dihydrochloride
$C_6H_5NO_2$	Picolinic acid
$C_9H_8O_4$	Caffeic acid
CAEAC	Caffeic Acid Equivalents Antioxidant Capacity
CCRD	Central Composite Rotatable Design
CE	Conventional Extraction
CO_2	Carbon Dioxide
CoF_2	Cobalt floride tetrahydrate
Cyclo-DOPA	Cyclo-3,4-dihydroxyphenylalanine
EFSA	European Food Safety Authority
EtOH	Ethanol
FDA	Food and Drug Administration
$FeCl_3$	Iron chloride
FL	Disodium fluorescein
H_2O	Water
H_2O_2	Hydrogen peroxide
HORAC	Hydroxyl Radical Adverting Capacity
HOSC	Hydroxyl Radical Scavenging Capacity
HPCDAE	High Pressure Carbon Dioxide-Assisted Extraction
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaH_2PO_4.H_2O$	Sodium phosphate monobasic monohydrate
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic dehydrate

ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffer Solution
PGSS®	Particles from Gas Saturated Solutions
PLE	Pressurized Liquid Extraction
POD	Peroxidase
PPO	Polyphenoloxidase
PVDF	Polyvinylidene difluoride
RSM	Response Surface Methodology
R^2	Correlation coefficient
R_{adj}^2	Adjusted correlation coefficient
SPB	Sodium Phosphate Buffer Solution
spp.	Specie
TBC	Total Betacyanin Content
TEAC	Trolox Equivalent Antioxidant Capacity
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
vs.	*Versus*

1 Introduction

1.1 Colorants in Food Industry

Food industry and industrial production of colorants have become very important over the past decades. Tons of colouring agents are used every day, frequently to enhance, improve or restore colour of food products during processing (Borges *et al.*, 2012; Cardoso-Ugarte *et al.*, 2014; Nemzer *et al.*, 2011; Vergara *et al.*, 2014).

Colorants are highly conjugated systems which absorbs electromagnetic radiation between wavelengths of 400 nm to 800 nm appear to be coloured. They produce colour when are added to a product (Cavalcanti *et al.*, 2013). Colour is one of the most important appearance attributes of foods, is the first prominent sensorial characteristic that influences the consumer selection of final product, being considered as a quality indicator (Azeredo, 2009; Boo *et al.*, 2012; Borges *et al.*, 2012; Jimenez-Aguilar *et al.*, 2011).

Figure 1.1. Natural pigments and their colours (Cavalcanti *et al.*, 2013).

Colouring agents can be defined by their origin as natural, synthetic or inorganic colorant. Natural colours are ubiquitous in nature however the natural colorants that are commercially available remain relatively low (Cardoso-Ugarte *et al.*, 2014). Natural pigments are defined as dyes or colorants obtained from natural sources, such as plants, animals and microorganisms. Nevertheless, the majority of commercial natural colorants currently used are extracted from plant sources such as roots, fruits, barks, leaves, wood, fungi and lichens

(Cavalcanti *et al.*, 2013; Delgado-Vargas *et al.*, 2000). Nature produces a variety of compounds adequate for food colouring (Figure 1.1.), such as carotenoids, chlorophylls, anthocyanins, betalains and carminic acid (Azeredo, 2009; Boo *et al.*, 2012).

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 (Boo *et al.*, 2012; de Paz *et al.*, 2012; Santos and Meireles, 2011; Shahid *et al.*, 2013). In addition, customer requirements are high, foods should contain ‘‘natural’’ ingredients. Furthermore, the safety of synthetic food colorants has been related to high levels of toxicity, allergic reactions, and carcinogenic potential (Azeredo, 2009; Boo *et al.*, 2012; Jimenez-Aguilar *et al.*, 2011; Maran *et al.*, 2013; Santos and Meireles, 2011; Shahid *et al.*, 2013). Although synthetic dyes have lower production costs and greater stability, the European Union and the United States have restricted their use as food additives. These restrictions have increased the use of natural pigments in the food industry (Borges *et al.*, 2012; Santos and Meireles, 2011).

Another major point concerning natural pigments is their biological activity, besides their technological function, the natural colours are found to be nutritional antioxidants and their presence in the diet can reduce the risk of cardiovascular diseases, cancer and diseases associated with ageing (Cavalcanti *et al.*, 2013; Maran *et al.*, 2013). Their health-benefit properties have been focused by many works, especially in case of carotenoids and anthocyanins. Betalains, because of their relative scarceness in nature, have been studied to a lesser degree as bioactive compounds (Azeredo, 2009; Tiwari and Cullen, 2013). Several studies have been dedicated to develop ways to improve the extractability and the stability of natural pigments (Cardoso-Ugarte *et al.*, 2014).

1.2 Betalains

The term betalain originates from the Latin name of beet root (*Beta vulgaris*), from which betalains were first extracted (Tiwari and Cullen, 2013). Betalains are present in most plants belonging to the order *Caryophyllales*, they fulfil the role of anthocyanins. They are responsible not only for the bright coloration of fruits and flowers, but also of roots and leaves of plants belonging to this order (Castellar *et al.*, 2006; Gandia-Herrero and Garcia-Carmona, 2013; Maran and Manikandan, 2012; Obon *et al.*, 2009).

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. Chemically, betacyanins are derivatives of betanidin containing a cyclo-3,4-dihydroxyphenylalanine (cyclo-DOPA) residue, whereas betaxanthins result from the condensation of α-amino acids or amines with betalamic acid (Figure 1.2.). The conjugation of betalamic acid with cyclo-DOPA residue shifts the absorption maximum from 480 nm (yellow, betaxanthins) to about 540 nm (violet, betacyanins) (Boo *et al.*, 2012; Castellar *et al.*, 2003; Delgado-Vargas *et al.*, 2000; Moreno *et al.*, 2008).

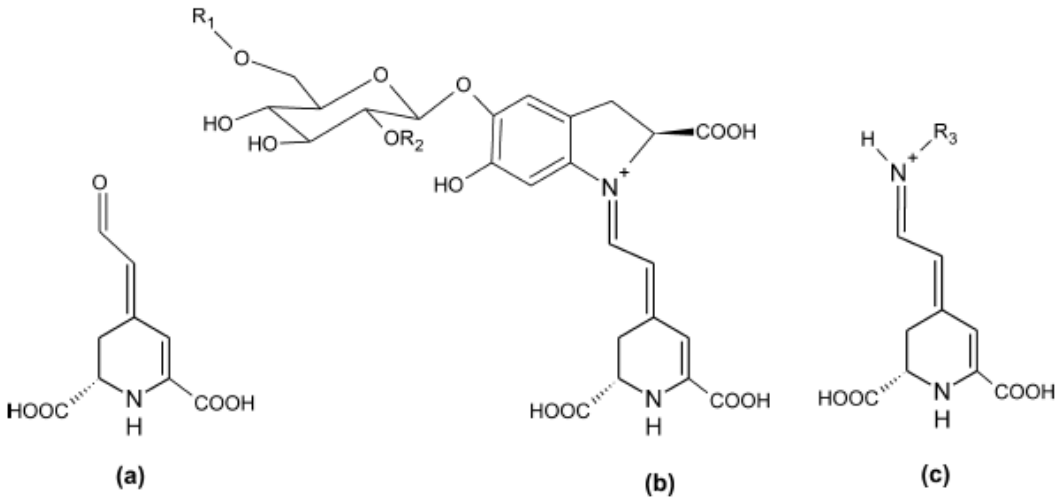

Figure 1.2. Chemical structure of (a) betalamic acid (b) betacyanins and (c) betaxanthins (Cavalcanti *et al.*, 2013).

To date, betalains comprise about 55 structures. The major components found in betacyanins and betaxanthins are betanidin and vulgaxanthine I and II, respectively (Cavalcanti *et al.*, 2013; Stintzing and Carle, 2007; Tiwari and Cullen, 2013). Although structurally related to alkaloids, betalains have no toxic effects in human health as can be deduced from the fact that they are present in high amounts in some foodstuffs. Therefore, they are considered a safe natural colorant source (Cavalcanti *et al.*, 2013; Delgado-Vargas *et al.*, 2000).

Besides their colorant proprieties, betalains have attracted much attention because of their bioactivities. These pigments are classified as antioxidants, i.e. compounds that stop or delay the oxidation processes. Consequently, they can be used in the treatment of inflammatory and cardiovascular diseases, cancer, asthma, arthritis, oxidative stress, intestinal inflammation, diabetes, and other diseases associated with aging. The added value of these pigments is

increased owing to their double function as colorant and as antioxidant (Allegra *et al.*, 2005; Butera *et al.*, 2002; Cai *et al.*, 2003; Castellar *et al.*, 2003; Khan *et al.*, 2012; Osorio-Esquivel *et al.*, 2011; Sanchez-Gonzalez *et al.*, 2013; Serra *et al.*, 2013; Stintzing and Carle, 2007; Stintzing *et al.*, 2005; Tesoriere *et al.*, 2005).

1.2.1 Natural Sources

Betalains are found in vacuoles of red beetroots, cactus fruits, foliage of chards, flowers of bougainvillea and *Amaranthus* plants. The richest source of betalains is red beetroots and their level of betalains among red beet varieties varies from 44 to 60 mg/100 g fresh weight (Tiwari and Cullen, 2013). 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 colorant is classified as additive E-162 (EU) and 73.40 (FDA, USA) (Azeredo, 2009; Castellar *et al.*, 2006; Gandia-Herrero and Garcia-Carmona, 2013; Gandia-Herrero *et al.*, 2010; Maran and Manikandan, 2012; Obon *et al.*, 2009). 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 (Castellar *et al.*, 2006; Gandia-Herrero *et al.*, 2010; Moreno *et al.*, 2008; Vergara *et al.*, 2014). Therefore, attempts have been made to search alternative sources of betalains.

Figure 1.3. *Opuntia* spp. fruits.

In this field, cactus pears fruit (Figure 1.3.) have been identified to be a promising 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 (Maran *et al.*, 2013; Saenz *et al.*, 2009). The largest genus of the *Cactaceae* family is mainly used for fruit production. It is known for rapid growth, good adaptation to poor

soils and low requirement for water. Its fruit, cactus pear fruit, prickly pears or Indian fig is a fleshy berry, varying in shape, size and colour has very tasty pulp full of seeds. These fruits are characterized by the presence of betalains, particularly betanin and indicaxanthin (Butera *et al.*, 2002; Maran *et al.*, 2013; Maran and Manikandan, 2012; Obon *et al.*, 2009; Saenz *et al.*, 2009)**.** Prickly pear cultivars contained total betalains up to 9 mg/100 g fresh weight (Butera *et al.*, 2002; Castellar *et al.*, 2003; Feugang *et al.*, 2006; Sanchez-Gonzalez *et al.*, 2013; Stintzing *et al.*, 2005; Tiwari and Cullen, 2013).

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 compound (Castellar *et al.*, 2006; Gandia-Herrero and Garcia-Carmona, 2013; Maran and Manikandan, 2012; Obon *et al.*, 2009). 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 (Azeredo, 2009; Castellar *et al.*, 2006; Castellar *et al.*, 2003; Gandia-Herrero and Garcia-Carmona, 2013; Maran and Manikandan, 2012; Obon *et al.*, 2009). The commercial exploitation of these fruit as alternative source of food colorants may contribute to the sustainable development of the underdeveloped semi-arid regions (Azeredo, 2009). Thus, there is an increasing interest for large-scale cactus pear fruit processing for the production of colouring foodstuffs, opening new markets on functional foods to food industries (Obon *et al.*, 2009).

1.2.2 Extraction Methodologies

Extraction is a method used for obtaining components from a solid mixture or solution. In recent years, extraction techniques have been explored toward increasing efficiency, reducing operating times and limiting the use of organic solvents, for the development of cheaper and ‘‘greener’’ analytical methodologies (Herrero *et al.*, 2013; Huang *et al.*, 2013).

Extraction of bioactive compounds from various plant sources can be done by various extraction procedures, namely conventional and non-conventional methods. Conventional techniques for the solvent extraction of bioactive compounds from natural matrices are based on the extraction power of different solvents coupled with the use of heat and/or agitation. These extraction methods include soxhlet extraction, maceration and hydrostillation (Azmir *et al.*, 2013; Tiwari and Cullen, 2013; Wang and Weller, 2006).

Betalains are mainly extracted through conventional extractions. Betalain-containing materials are generally macerated or ground. Extraction of pigments is commonly performed with water, although, in some cases, the use of methanol or ethanol solutions (20–50% v/v) is necessary to complete extraction (Azeredo, 2009; Delgado-Vargas *et al.*, 2000; Maran *et al.*, 2013; Maran and Manikandan, 2012; Sanchez-Gonzalez *et al.*, 2013; Tiwari and Cullen, 2013). Nevertheless, the aqueous extractions promote better stability of these pigments. Slight acidification of the extraction medium is recommended, to enhance betacyanin stability and avoids possible oxidation by polyphenoloxidase (PPO) activity (Azeredo, 2009; Strack *et al.*, 2003).

Traditional extraction methods, used to obtain this type of compounds, 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 (Azmir *et al.*, 2013; Borges *et al.*, 2012; Herrero *et al.*, 2006; Huang *et al.*, 2013; Santos and Meireles, 2011; Tiwari and Cullen, 2013; Xu *et al.*, 2010). Therefore, there is a growing demand for developing suitable extraction techniques that improve process efficiency through enhanced mass transfer and which are more environmentally friendly (Tiwari and Cullen, 2013; Xu *et al.*, 2010).

Presently, extraction methodologies able to overcome the disadvantages mentioned above are being studied. These techniques are referred as non-conventional extraction techniques. Among them, Pressurized Liquid Extraction (PLE) and High Pressure Carbon Dioxide-Assisted Extraction (HPCDAE) could be used to obtain antioxidant pigment-rich extracts from biological materials. These extraction techniques provide higher selectivity, shorter extraction times and do not use toxic organic solvents (Borges *et al.*, 2012; Herrero *et al.*, 2006; Santos and Meireles, 2011; Xu *et al.*, 2010).

PLE is one of the called green technologies (Azmir *et al.*, 2013; Borges *et al.*, 2012). This technique is also known as pressurized liquid extraction, pressurized solvent extraction, accelerated solvent extraction, enhanced solvent extraction and high pressure solvent extraction (Azmir *et al.*, 2013; Mustafa and Turner, 2011). The basic principle of PLE relies on the combination of high pressure and temperature in order to modify the properties of the solvents, namely density, diffusivity, viscosity and dielectric constant, allowing the selection of types of extracted compounds according to their polarity. By applying those conditions, faster extraction processes result in higher extraction yields with small amounts of solvents (Azmir *et al.*, 2013; Borges *et al.*, 2012; Herrero *et al.*, 2013; Mustafa and Turner, 2011).

PLE is suitable for a wide range of solutes, polar to non-polar. The type of solvent, extraction time, temperature, particle size and water content of the sample are the factors that mostly affect this extraction process (Mustafa and Turner, 2011). Temperature is one of the most important parameters for PLE. Higher extraction temperature can promote higher analyte solubility and also decrease the viscosity and surface tension of solvents, allowing a better penetration of the solvent into the matrix (Azmir *et al.*, 2013; Herrero *et al.*, 2013; Mustafa and Turner, 2011; Sun *et al.*, 2012). In PLE, pressure is another significant parameter which may influence compounds recovery. The main advantage of applying pressure during the extraction process is to keep the solvent in a liquid state at elevated temperatures, above the boiling point of the solvent. The use of elevated pressure and temperature reduce solvent surface tension, forcing the solvent within the matrix pore to contact the analyte and in this way making the analyte more available. Furthermore, depending on the location of the analyte within the matrix, high pressure could result in the disruption of the plant tissue, cellular wall, membrane and organelles, increasing its permeability and enhancing the mass transfer of the solvents into the matrix and the soluble constituents into the solvent used in extraction (Gil-Chavez *et al.*, 2013; Huang *et al.*, 2013; Mustafa and Turner, 2011; Santos and Meireles, 2011; Sun *et al.*, 2012).

Another important method is HPCDAE that consists in a solvent extraction assisted with pressurized carbon dioxide. HPCDAE combines the advantages of enhanced mass transfer rates increasing compounds diffusion from the vegetable matrix into the selected solvent extraction. Carbon dioxide (CO_2) is a nontoxic, nonflammable, non-explosive and inexpensive agent. It is easily removed simply by depressurization and out-gassing (Hu *et al.*, 2013; Santos and Meireles, 2011; Xu *et al.*, 2010). In addition, this molecule is environmentally friendly and "generally recognized as safe" (GRAS) by FDA (U.S. Food and Drug Administration) and EFSA (European Food Safety Authority). Moreover, CO_2 has been described to ensure minimal alteration of the bioactive compounds and to preserve their functional properties (Gil-Chavez *et al.*, 2013).

Like many other natural pigments, betalains are extremely sensitive to oxidation especially that caused by peroxidase (POD) activity, which is one of the main causes of discoloration of this pigment. When betalains are effluxed from their cellular compartment, the vacuoles, come in contact with enzymes, mainly POD and PPO, which quickly degrade the pigments. To avoid betalain enzymatic degradation, the enzyme can be effectively inactivated by a short heat treatment of the extract (70 ºC, 2 min), although this may degrade some of the pigments and loss another nutritional components (Azeredo, 2009; Delgado-Vargas *et al.*, 2000; Liu *et al.*, 2008; Liu *et al.*, 2010; Tiwari and Cullen, 2013).

To overcome the disadvantages mentioned above, pressurized CO_2 might be an alternative to inactivate not only enzymes but also pathogens. Pressure alone has no direct influence on enzyme at pressures below 20 MPa (Hu *et al.*, 2013). The effect of pressurized CO_2 is demonstrated to disrupt bacterial cells by the rapid release of gas pressure; numerous studies have showed the efficacy of pressurized CO_2 to inactivate microorganisms and enzymes in batch, semi-continuous, and continuous systems (Liu *et al.*, 2008; Liu *et al.*, 2010; Wimmer and Zarevucka, 2010; Xu *et al.*, 2010). Limited studies in literature regarding the effect of HPCD on quality of betalain-containing extracts are available. Liu *et al.* (2008) reported that no significant change was found in the colour shade of red beet extract by HPCD treatment.

In this field, HPCD besides microorganisms and enzymes inactivation might also strengthened extraction process by its superior abilities in disruption of the plant tissue, cell membrane modification, intracellular pH decrease, disordering of the intracellular electrolyte balance and removal of vital constituents from cells (Hu *et al.*, 2013; Xu *et al.*, 2010). However, there is no study on HPCDAE of betalains to date.

1.2.3 Microencapsulation Techniques

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 (Gandia-Herrero and Garcia-Carmona, 2013). The stability is an important aspect to consider for the use of these pigments as colorants in foods. Betalains stability is affected by many factors, such as temperature, pH, oxygen, exposure to light, aqueous activity and enzymatic activities. Moreover, temperature is the most decisive factor for betalain decomposition (Diaz-Reinoso *et al.*, 2006; Janiszewska, 2014; Saenz *et al.*, 2009). The stabilization of betalains could be improved using microencapsulation technologies.

Microencapsulation is described as a technique where in a bioactive compound is encapsulated in a biopolymer. One of the objective of microencapsulation is to increase the shelf life of the bioactive compound, protecting it from undesirable environmental conditions (among others light, moisture and oxygen) thereby reducing its reactivity to its outside environment (Janiszewska, 2014; Ravichandran *et al.*, 2012; Saenz *et al.*, 2009). This method is also used to change liquid solutions to powders, which are easier to handle (Kandansamy and Somasundaram, 2012; Saenz *et al.*, 2009).

One of the most widely used techniques of microencapsulation is spray drying. The rapid drying of the solutions leads to the formation of encapsulated form in which the colorant is surrounded by a wall material (Janiszewska, 2014). Recent studies have described preparation of spray-dried hydrophilic formulations containing betalains from red beet, cactus pear and amaranth (Cai and Corke, 2000; Gandia-Herrero and Garcia-Carmona, 2013; Gandia-Herrero *et al.*, 2010; Obon *et al.*, 2009; Pitalua *et al.*, 2010; Saenz *et al.*, 2009). However, the use of these hydrophilic forms to colour food lipophilic matrices is very limited, due to solubility issues.

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 lipidic 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 (de Paz *et al.*, 2012). The PGSS® process is especially suited for processing polymers and lipids in which CO_2 has a large solubility and a melting depression effect (Lack *et al.*, 2005). 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 (Weidner, 2009). Within this context, PGSS® methodology seems to be an alternative to the conventional precipitation processes for the development of lipidic particles of betalains.

1.3 Aim and rational of the thesis

This thesis focuses on the development of adequate clean and mild methodologies for isolate and stabilize of betacyanins, from cactus pear fruits (*Opuntia* spp.), with potential application as natural colorant in food industry.

To achieve this goal, an integrated approach was developed by: i) exploiting different environmentally and friendly extraction methodologies to isolate these natural pigments, improving their extraction yields with minimal degradation, and ii) modelling the use of supercritical fluid technology to develop lipidic particles of a natural betacyanin-rich extract.

Within this context, the work was divided into two majors parts, as schematically presented in Figure 1.4.

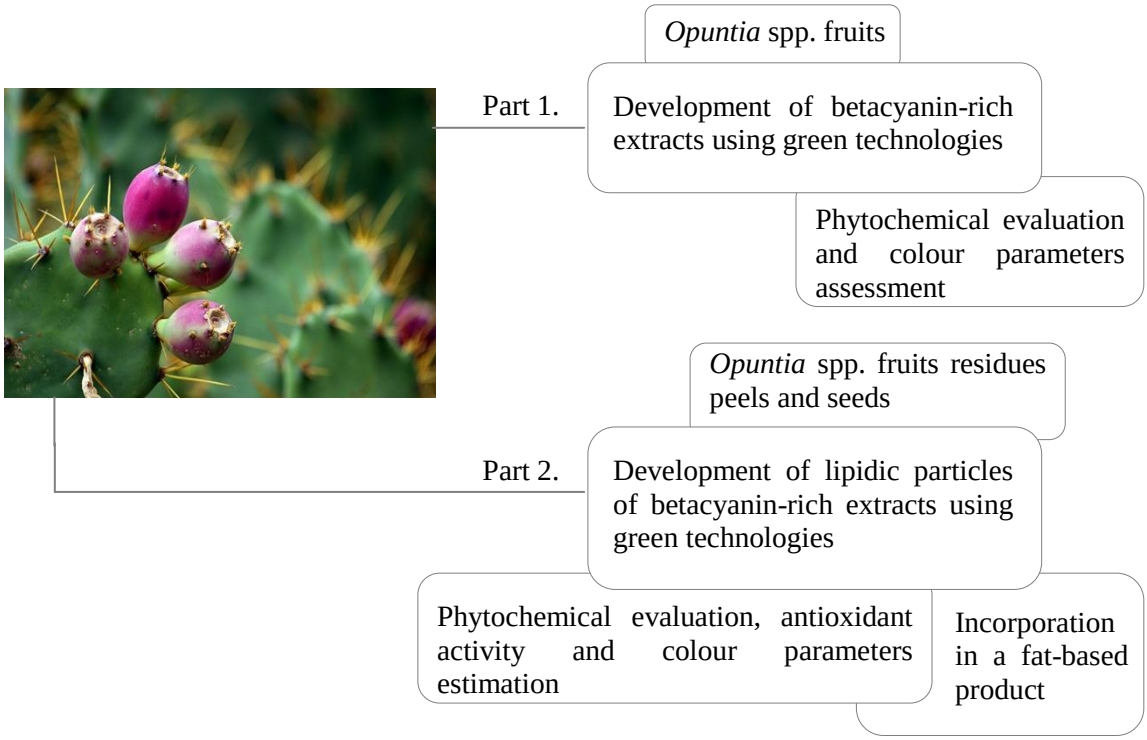

Figure 1.4. Structure of the thesis.

In Part 1, two different extraction technologies, namely PLE and HPCDAE, were exploited to isolate of betacyanins form *Opuntia* spp. fruits. All developed *Opuntia* spp. extracts were characterized in terms of phytochemical composition and colour parameters.

In Part 2, *Opuntia* spp. juice residues (peels and seeds) were conventionally extracted. Supercritical Fluid Technology, namely PGSS®, was exploited to develop lipidic particles of this conventional extract derived from *Opuntia* spp. juice residues. The developed lipidic particles were evaluated in terms of global yield, phytochemical composition, antioxidant activity, colour parameters and particle morphology. The *Opuntia* spp. extract and lipidic particles were incorporated in a fat-based product.

2 Experimental procedure

2.1 Chemicals

Chemicals used for different extractions methodologies were: Carbon dioxide 99.95% from Air Liquide (Lisbon, Portugal), ethanol absolute 99.9% from Scharlau (Barcelona, Spain), distillated water and citric acid from Sigma- Aldrich (St Quentin Fallavier, France).

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

For phytochemical characterization: sodium carbonate (Na_2CO_3) were 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 floride 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).

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).

2.2 Extraction of Betalains from *Opuntia* spp. fruits

2.2.1 Cactus pear (*Opuntia* spp.) harvesting

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 - N37º04.400, W008º06.100). All prickly pears fruits were harvested at comparable ripening stages (physiological maturity).

2.2.2 Fruits samples preparation

For sample preparation, spikes were removed with a brush and *Opuntia* spp. fruits were freeze dried at -100 ºC, 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.

2.2.3 *Opuntia* spp. fruits extraction

2.2.3.1 Conventional Extraction (CE)

Opuntia spp. fruits were extracted in the dark with acidified H_2O pH 5.0 by citric acid (ratio 1:480, w/v), for 2 hours at 45 ºC and 200 rpm (IKA® dual-speed mixer RW 20.n, Staufen, Germany). The extracts were then centrifuged at 9000 rpm for 10 minutes and the supernatants were concentrated in a rotary evaporator at 40 ºC and under reduced pressure (23 mbar) until having a concentration of 25 g/L. The resulting extracts were kept in a cold, dry and dark environment until further analyses.

2.2.3.2 Pressurized Liquid Extraction (PLE)

The extractions were carried out in a supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SFE-500F-2-C50) comprising a 500 mL cylinder extraction cell (extraction vessel) and two different separators (fraction collector 1 and fraction collector 2), each of them with 500 mL of capacity, with independent control of temperature and pressure. This apparatus is schematically represented in Figure 2.1.

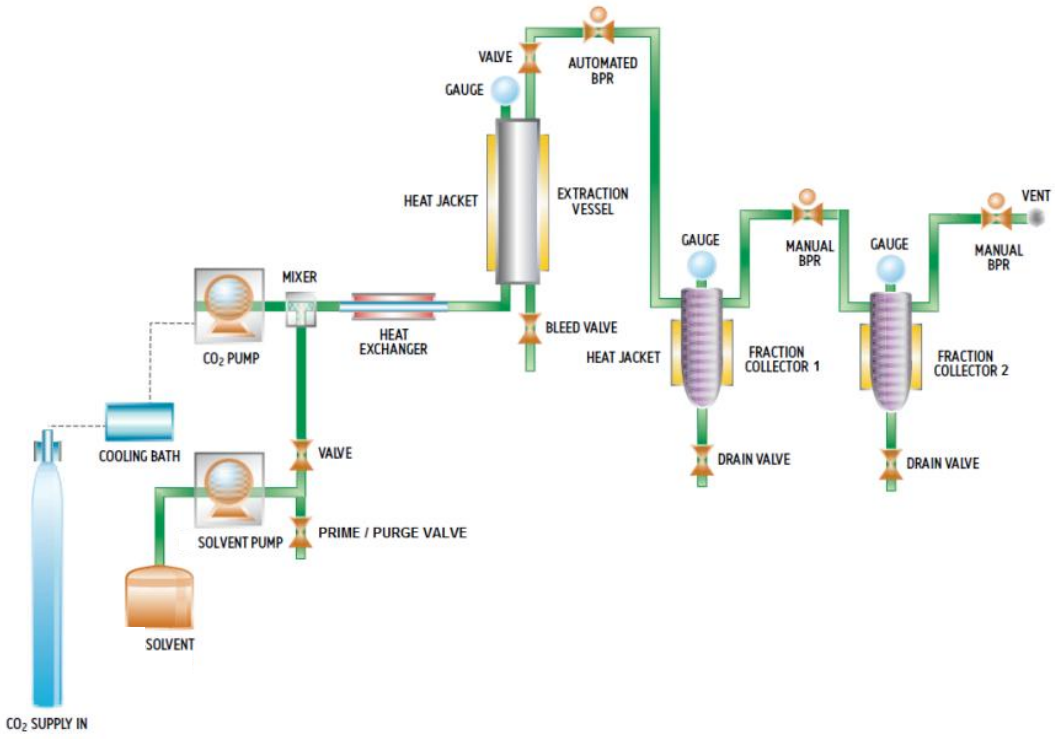

Figure 2.1. Schematic diagram of the pressurized extraction apparatus (Adapted from Waters, 2010).

In dynamic mode, the extraction vessel was filled with about 5 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 15-25 MPa. The solvent was preheated on a heat exchanger to a temperature of 25-45 ºC. 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 20 g/min. Samples were collected at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 minutes in plastic flaks.

In static mode, the extraction vessel was filled with about 1 g of dried *Opuntia* spp. fruits. 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) and preheated on a heat exchanger to a temperature of 25-45 ºC. The vessel was pressurized to the required pressure, 15-25 MPa, and held to the required extraction time, 120 minutes. After PLE, aqueous extracts were rapidly cooled to 5 ºC in ice water to prevent betacyanins degradation. All aqueous extracts were concentrated in a rotary evaporator (BUCHI Rotavapor R-210, Flawil,

Switzerland) at 40 ºC and under reduced pressure (23 mbar) until having a concentration of 25 g/L. The resulting extracts were kept in a cold, dry and dark environment until further analyses.

2.2.3.3 High Pressure Carbon Dioxide Assisted-Extraction (HPCDAE)

The extractions were carried out in the same extractor (Thar Technology, Pittsburgh, PA, USA, model SFE-500F-2-C50). For each experiment, the extraction vessel was filled with about 1 g of dried *Opuntia* spp. fruits. Liquid carbon dioxide was delivered to the extraction vessel using a TharSFC P-50 high pressure pump (Thar Technology, Pittsburgh, PA, USA) and acidified distilled water pH 5.0 by citric acid was delivered by a second similar high pressure pump. The solvents, carbon dioxide and water (25:75, 50:50; 75:25, % v/v), were then combined on a mixer and preheated on a heat exchanger to a temperature of 25-45 ºC. The vessel was pressurized with the mixture of solvents to the required pressure, 15-25 MPa, and held to the required extraction time, 120 minutes. Then the depressurization was performed by releasing CO_2 into the atmosphere. After completion of HPCD Assisted-Extraction, the aqueous extract was collected into a plastic flak immersed in ice water (5 °C) to prevent betacyanin and phenolic compounds degradation. All aqueous extracts were concentrated in a rotary evaporator evaporator (BUCHI Rotavapor R-210, Flawil, Switzerland) at 40 ºC and under reduced pressure (23 mbar) until having a concentration of 25 g/L. The resulting extracts were kept in a cold, dry and dark environment until further analyses.

2.2.4 Characterization of *Opuntia* spp. Extracts

2.2.4.1 Total betacyanin content

The total betacyanin content of the *Opuntia* spp. extracts was calculated according to (Guzman-Maldonado *et al.*, 2010). All extracts were spectrophotometrically measured at 476, 538, and 600 nm. Betacyanins and betaxanthins were determined with Nilsson equations:

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

$$\%\, Betaxanthins = \frac{y}{750} \times DF \times 100$$

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 (mg/100 g) and were expressed as a mean of triplicates.

2.2.4.2 Colour parameters

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 $h^\circ = \arctan(b^*/a^*)$. The colour parameters were expressed as a mean of triplicates. These values were then converted to RGB (Red, Green, and Blue *color* values), using the software *OpenRGB* (Logicol).

2.3 Microencapsulation of betalain-rich *Opuntia* spp. extract

2.3.1 Cactus pear (*Opuntia* spp.) harvesting

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 prickly pears fruits were harvested at comparable ripening stages (physiological maturity).

2.3.2 Fruits samples preparation

Opuntia spp. fruits samples were prepared as described 2.2.2.

2.3.3 *Opuntia* spp. juice residues Conventional Extraction (CE)

Opuntia spp. 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 2 hours at room temperature and 200 rpm (IKA® dual-speed mixer RW 20.n, Staufen, Germany). The extracts were then centrifuged at 9000 rpm for 10 minutes and the supernatants were concentrated in a rotary evaporator (BUCHI Rotavapor R-210, Flawil, Switzerland) at 40 ºC and under reduced pressure (58 mbar). After the removal of the alcoholic fraction, the extracts were freeze dried at -20 ºC (FreeZone Plus 4.5 L Cascade Freezer Dry System, LABCONCO®, Kansas City, United States of America), in the absence of light, during 48 hours. The resulting extracts were kept in a cold, dry and dark environment until further analyses.

2.3.4 Emulsion preparation

Water-in-Oil (W/O) emulsion was prepared by melting previously 1.56 g of lipid (Glyceryl monoostearate), and adding 0.06 g of emulsifier (Polyglyceryl-3 Polyricinoleate). The dried *Opuntia* spp. extract was dissolved in distilled water (1300 g/L). Then, this aqueous phase containing betacyanin extract 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.

2.3.5 Experimental design (Process Optimization)

Response Surface Methodology (RSM) was used to model the encapsulation of betacyanins and optimize encapsulation conditions. RSM consists of a set of mathematical and statistical methods developed for modelling phenomena and finding combinations of a number of experimental factors (variables) that will lead to optimum responses. With RSM, several variables are tested simultaneously with a minimum number of trials, using special experimental designs that enable to find interactions between the variables which cannot be identified with classical approaches. The encapsulation of betacyanins through PGSS® was 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 carried out: 8 factorial points (coded levels as (+1) and (-1); 6 star points (coded as (+α) and (-α)); 3 centre points (coded as 0) (Table 2.1.).

The pressure varied from 9 to 20 MPa, the temperature from 55 to 70 ^{0}C and the equilibrium time from 5 to 5 min, according to the experimental design followed (Table 2.2.). 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 2.3.6.

Table 2.1. Independent variables and their levels used in the Central Composite Design.

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

Table 2.2. Design of Experiments: Central Composite Design methodology.

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)

2.3.6 Precipitation by Particles from Gas Saturated Solutions (PGSS®)

Lipidic particles of *Opuntia* spp. extract were produced using the PGSS® technique. The schematic representation of the modified PGSS® equipment (Separex Supercritical & High Pressure Technology) used to produce the particles is shown in Figure 2.2. 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 a 18 L collector vessel at atmospheric pressure.

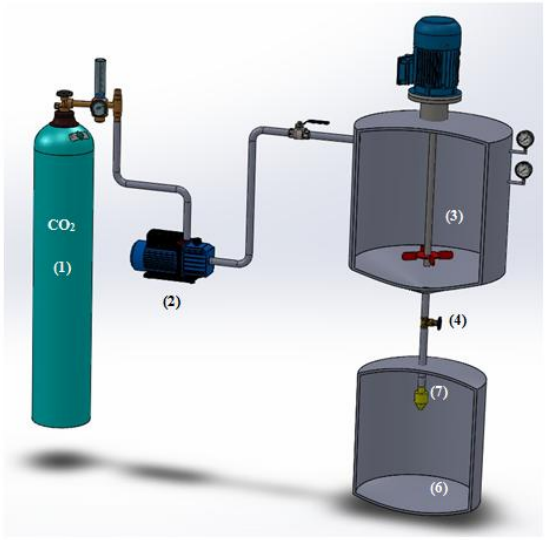

Figure 2.2. Schematic diagram of the PGSS® apparatus: (1) CO_2 cylinder (2) pneumatic piston pump (3) stirred vessel (electrically thermostated) (4) automated depressurisation valve (6) recovery vessel (7) nozzle.

2.3.7 *Opuntia* spp. extract and particle characterization

2.3.7.1 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.

2.3.7.2 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.

2.3.7.3 Colour parameters

The colour of *Opuntia* spp. dried extract and *Opuntia* spp. particles were assessed by CIElab method as described in 2.2.4.2.

2.3.7.4 Total betacyanin content

The measurement of betacyanin content of the *Opuntia* spp. extract and particles was performed as described at 2.2.4.1. To ensure the total destruction of the particles and the total release of the betacyanins, all the samples analyzed were previously dissolved in water (40 g/L) by ultrasonication extraction and filtered through a PVDF membrane (0.45 µm pore size).

2.3.7.5 Antioxidant activity

For the antioxidant activity assays, the *Opuntia* spp. lipidic particles were pre-treated according to the method described in 2.3.7.4.

2.3.7.5.1 Oxygen Radical Absorbance Capacity (ORAC)

ORAC assay was carried out by the method of Huang *et al.* (2002) modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by Feliciano *et al.* (2009). 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 incubate 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 a blank. Solutions of 5, 10, 20, 30, and 40 µmol/L of Trolox were used as control standards. 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.

2.3.7.5.2 Hydroxyl Radical Adverting Capacity (HORAC)

The HORAC assay was based on a previously reported method (Ou *et al.*, 2002) 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 ($9.28x10^{-8}$ 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. 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.

2.3.7.5.3 Hydroxyl Radical Scavenging Capacity (HOSC)

The HOSC assay was performed according to (Moore *et al.*, 2006) 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. Data was expressed as µmol of trolox

equivalents antioxidant capacity (TEAC) per g of particles. Results were presented as a mean of eight replicates.

2.4 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 1.75 mg/L. The mixtures were freeze and analyzed visually, concerning the colour homogenization.

2.5 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™, 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) (Gacula and Singh, 1984; Haaland, 1989). The R^2 value provides a measure of how much of the variability in the observed response values can be explained by the experimental factors and their interactions. However, the R^2 should be used with caution since it always increases with the inclusion of a new variable in the model. The use of R_{adj}^2 is preferred and is related with R^2 by the following equation (Weisberg, 1985):

$$R_{adj}{}^2 = 1 - \frac{n-1}{n-p}(1-R^2)$$

Where n is the number of experiments and p is the number of variables (factors) in the model. The $R_{adj}{}^2$ takes into account the fact that the number of residual degrees of freedom in the polynomial regression changes as the order of the polynomial changes. $R_{adj}{}^2$ is an unbiased estimate of the coefficient of determination and is always smaller than R^2. In practice, R^2 should be at least 0.75 or greater; values above 0.90 are considered to be very good (Haaland, 1989).

3 Results and discussion

Cactus pears (*Opuntia* spp.) have been identified to be a promising betalainic crop, covering a wide coloured spectrum from yellow to purple pigments, with potential application as natural colorant in food industry.

Firstly, two different environmentally and friendly extraction technologies, namely PLE and HPCDAE, were exploited to isolate betacyanins form *Opuntia* spp. fruits, harvested in Quarteira, Portugal. All *Opuntia* spp. extracts were characterized in terms of global yield, phytochemical composition and colour parameters (section 3.1).

Afterward, *Opuntia* spp. juice by-products (fruit peels and seeds) were conventionally extracted. Supercritical Fluid Technology, namely PGSS®, was exploited to develop lipidic particles of this conventional extract derived from *Opuntia* spp. juice by-products. This extract was characterized in terms of phytochemical composition, antioxidant activity and colour parameters for further comparison with the commercial red beet pigment (section 3.2.1). The developed lipidic particles were evaluated in terms of global yield, particle morphology, colour parameters, phytochemical composition and antioxidant activity (section 3.2.2). Finally, the incorporation of *Opuntia* spp. extract (obtained from residues) and lipidic particles was evaluated in a fat-based product (3.2.3).

3.1 Extraction of betacyanins from *Opuntia* spp. fruits

In this work, two different emerging novel technologies, namely PLE and HPCDAE, were exploited to isolate betacyanins from *Opuntia* spp. fruits. Furthermore, PLE and HPCDAE variables such as extraction pressure, temperature and mass ratio of pressurized CO_2 *vs*. acidified water (CO_2:H_2O) were the extraction parameters studied for the maximization of betacyanin content. In order to define the extraction media, some previous studies on betacyanins extraction from *Opuntia* spp. were taken into account. In one of the studies, Castellar *et al.* (2003) concluded that maximum stability of the *Opuntia* spp. pigments was achieved at pH 5. Furthermore, the water used in all extractions experiments was acidified until pH 5 using citric acid. The experimental conditions, yield and total betacyanin content (TBC) of *Opuntia* spp. fruits extractions using PLE (dynamic and static mode) and HPCDAE are found in Table 3.1.

Table 3.1. Experimental conditions, global yield and total betacyanin content of *Opuntia* spp. extracts using PLE and HPCDAE.

	CO_2:H_2O (%, w/w)	Pressure (MPa)	Temperature (°C)	Extarction time (h)	Global yield[a] (%, g/g)	TBC[b] (mg/100g fw)
PLE dynamic	0:100	15	25	2	44	13.2 ± 0.1
	0:100	15	45	2	44	13.6 ± 0.5
	0:100	25	25	2	49	17.7 ± 0.4
	0:100	25	45	2	50	14.7 ± 0.2
PLE static	0:100	15	25	2	23	8.0 ± 0.3
	0:100	15	45	2	32	12.1 ± 0.8
	0:100	25	25	2	48	16.0 ± 0.2
	0:100	25	45	2	47	14.5 ± 0.2
HPCDAE	25:75	15	25	2	47	17.8 ± 0.1
	25:75	15	45	2	54	22.8 ± 1.1
	25:75	25	25	2	49	19.7 ± 0.5
	25:75	25	45	2	59	21.4 ± 0.8
	50:50	15	45	2	48	16.4 ± 0.9
	75:25	15	45	2	42	14.6 ± 0.0

[a] in terms of dry weight (%, g/g)
[b]Total betacyanin content (expressed as mg of betacyanin/100 g of fresh weigh of fruit)

From the results, the global yield of extraction ranges between 23-59% among the extraction methodologies explored. The maximum yield was obtained for HPCDAE with a ratio CO_2:H_2O of 25:75, at 15 MPa and 45 ºC.

As can be seen in Table 3.1., the total betacyanin content extracted from cactus pear through different methodologies ranges from 8.0 to 22.8 mg of betacyanin per 100 g of fresh weigh of fruit. Other authors have reported similar values (between 5 and 100 mg/100 g fresh fruit), in different cactus pear species (Castellar *et al.*, 2003; Feugang *et al.*, 2006; Sanchez-Gonzalez *et al.*, 2013; Stintzing *et al.*, 2005).

Figure 3.1. shows the total betacyanin content obtained for HPCDAE and PLE in static mode and the kinetic curves of betacyanin content for PLE in dynamic mode, in terms of dry weight of extracts.

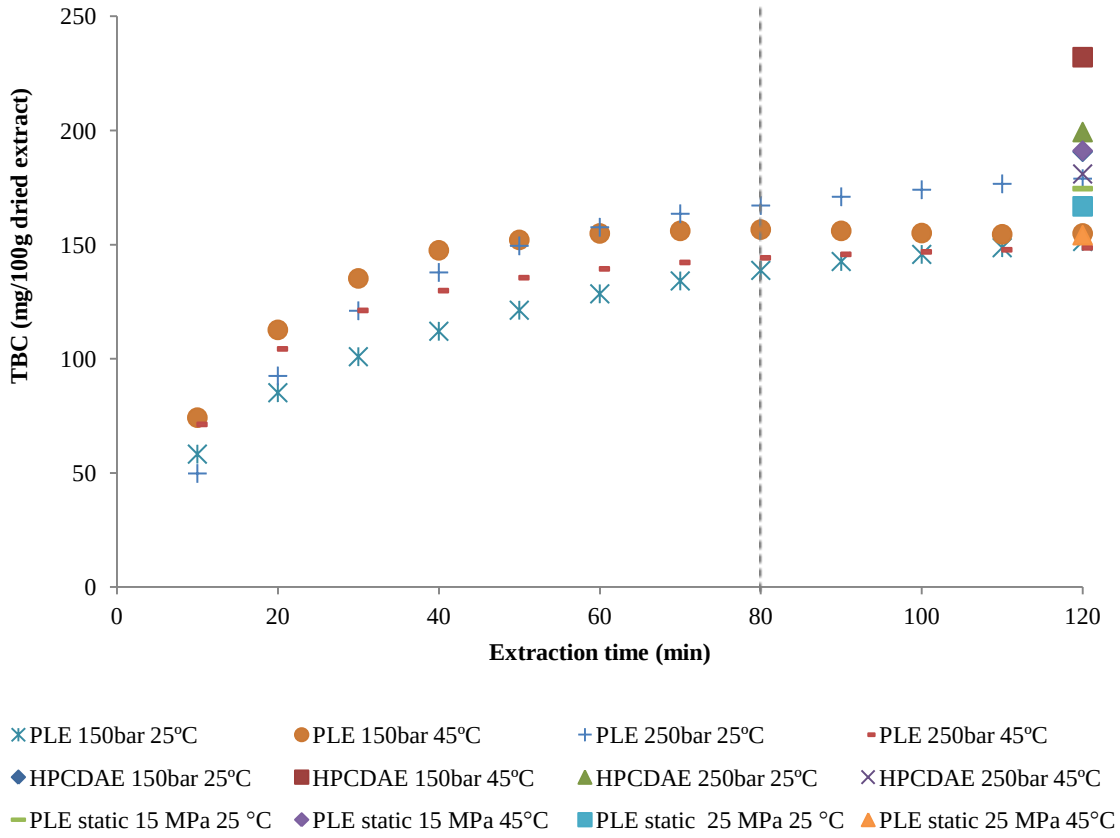

Figure 3.1. Yield of betacyanins of *Opuntia* spp. extracts obtained by PLE and HPCDAE.

All the experimental extraction kinetics curves regarding PLE in dynamic mode are clearly divided in two distinct zones. In the first zone, the yield increases with increasing extraction time, indicating a faster solubility of betacyanins into the extraction media. In the second zone, the yield was maximized into the steady-state yield, indicating that mobility of betacyanins from *Opuntia* spp. fruits into the extraction media approaches zero in the remaining time. In conclusion, extraction time of 80 minutes might be sufficient to achieve the same final betacyanin content obtained after 120 minutes.

From Figure 3.1., the total betacyanin content, obtained for different extraction methodologies, ranges from 148 to 211 mg of betacyanin per 100 g of dried extract. The highest betacyanin content was achieved for HPCDAE at 15 MPa and 45 ºC, whereas the lowest one was obtained for PLE in static mode at 15 MPa and 25 ºC.

The betacyanin content of all extraction methodologies, using different extraction conditions of temperature and pressure is present in Figure 3.2. The impact of process variables on the extraction of betacyanins was noticeable from the results. As expected, an increase in pressure and temperature results in an enhancement of betacyanins recovery from *Opuntia* spp.

fruits. Moreover, these extraction parameters had a strong influence on the water properties as a solvent. Higher pressures and temperatures result in the disruption of cell vacuole, making betacyanins more available. Moreover, these conditions also reduce water viscosity and surface tension, enhancing its penetration in the matrix and allowing faster betacyanins dissolution in water. To date, there is no study on literature about PLE and HPCDAE applied to betacyanins extraction.

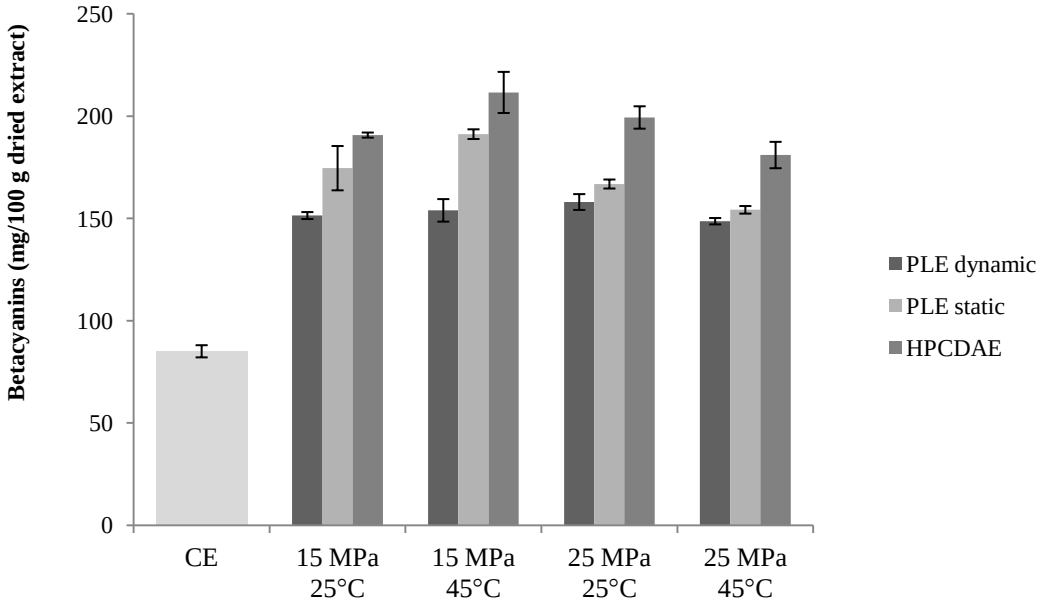

Figure 3.2. Impact of extraction pressure and temperature on recovery of betacyanins from *Opuntia* spp. fruits using PLE and HPCDAE.

The promising effect of the use of pressurized CO_2 for betacyanin extraction seems irrefutable when the results obtained using this technique are compared to that obtained using other extraction methods. Comparing CE with PLE and HPCDAE at same extraction conditions, it is possible to conclude that the last was more efficient in extracting betacyanins from *Opuntia* spp. fruits.

Figure 3.3. presents the betacyanin content for *Opuntia* spp. extracts using CE and HPCDAE at 15 MPa and 45 ºC, with different mass ratio of pressurized CO_2 *vs*. acidified water. It is possible to see that the mass ratio of pressurized CO_2 *vs*. acidified water at the optimum HPCDAE conditions had a stronger impact on betacyanins extraction. Higher betacyanins content were achieved with increasing ratio.

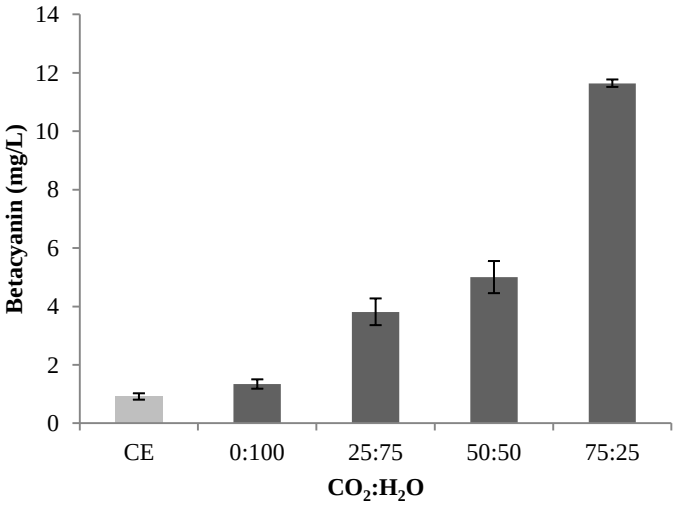

Figure 3.3. Effect of different mass ratio of pressurized CO_2 *vs*. acidified water in optimum HPCDAE conditions, 15 MPa and 45 ºC.

According to Santos and Meireles, (2011) and Xu *et al.* (2010), there are five possible forms associated with CO_2 in a HPCDAE, 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 HPCDAE of betacyanins. Firstly, supercritical CO_2 combines high diffusivity of gas with solvent strength of liquids with non-polar and lipophilic proprieties to dissolve phospholipid layer of cell membranes, improving the penetration of water into the cellular matrix and efflux of betacyanins from cell vacuoles to the outside of the cell. Secondly, the generation of *in situ* carbonic acid, when the CO_2 is added into HPCDAE system, decrease pH. This leads to a positive impact on betacyanins extraction and stability from *Opuntia* spp. fruits. Finally, the explosive effect during the rapid CO_2 depressurization causes disruption of cell vacuoles, making betacyanins more available enhancing extraction efficiency.

In addition, the effect of pressurized carbon dioxide has been reported to inactivate microorganisms and inhibit enzyme activity (PPO and POD), which are responsible for betacyanins degradation. 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 affects betacyanins stability (Santos and Meireles, 2011; Xu *et al.*, 2010). Overall, these advantages had contributed for the higher yields of betacyanins using HPCDAE.

The colour parameters of the *Opuntia* spp. extracts was measured using CIELab method. The results are presented in Table 3.2.

Table 3.2. Betacyanin content and colour parameters of *Opuntia* spp. extracts and commercial colourant.

	CO_2:H_2O (%, w/w)	Pressure (MPa)	Temperature (°C)	Extraction time (h)	TBC (mg/100 g)	L*	a*	b*	h°	C*	Colour conversion to RGB
PLE dynamic	0:100	15	25	2	151.4 ± 1.7	41.34 ± 0.11	76.28 ± 0.07	16.81 ± 0.03	0.21 ± 0.00	78.11 ± 0.08	
	0:100	15	45	2	153.9 ± 5.5	46.36 ± 0.05	81.51 ± 0.03	13.44 ± 0.02	0.16 ± 0.00	82.61 ± 0.03	
	0:100	25	25	2	158.0 ± 3.9	42.15 ± 0.03	79.71 ± 0.01	15.21 ± 0.03	0.18 ± 0.00	81.15 ± 0.01	
	0:100	25	45	2	148.6 ± 1.5	44.76 ± 0.04	78.78 ± 0.15	13.73 ± 0.05	0.17 ± 0.00	79.97 ± 0.14	
PLE static	0:100	15	25	2	174.6 ± 10.8	46.19 ± 0.06	71.54 ± 0.03	19.74 ± 0.14	0.26 ± 0.00	74.22 ± 0.03	
	0:100	15	45	2	191.2 ± 2.4	37.26 ± 0.05	73.97 ± 0.12	26.26 ± 0.02	0.34 ± 0.00	78.49 ± 0.11	
	0:100	25	25	2	166.8 ± 2.2	38.87 ± 0.02	67.20 ± 0.02	18.26 ± 0.02	0.26 ± 0.00	69.64 ± 0.01	
	0:100	25	45	2	154.2 ± 1.8	42.20 ± 0.03	77.41 ± 0.00	18.17 ± 0.04	0.23 ± 0.00	79.51 ± 0.04	
HPCDAE	25:75	15	25	2	190.7 ± 1.2	35.54 ± 0.03	71.62 ± 0.03	25.42 ± 0.02	0.34 ± 0.00	75.99 ± 0.02	
	25:75	15	45	2	211.6 ± 10.0	30.39 ± 0.01	64.43 ± 3.45	24.69 ± 0.05	0.36 ± 0.02	69.00 ± 3.22	
	25:75	25	25	2	199.4 ± 4.5	34.78 ± 0.01	71.81 ± 0.04	23.77 ± 0.06	0.31 ± 0.00	75.64 ± 0.02	
	25:75	25	45	2	181.0 ± 6.4	38.15 ± 0.35	72.25 ± 0.43	22.24 ± 0.08	0.29 ± 0.00	75.59 ± 0.43	
Commercial colourant											
E-162, liquid (Castellar *et al.*, 2006)	n.i.	n.i.	n.i.	n.i.	n.i.	69.5 ± 0.1	57.9 ± 0.2	- 1.0 ± 0.0	359	57.9	

n.i.: not indicate; TBC: Total Betacyanin content

From the results in Table 3.2., lightness ranged from the L^* = 30.39 of *Opuntia* spp. extracted by HPCDAE at 15 MPa and 45 ºC, to the L^*= 46.36 of *Opuntia* spp. extracted by PLE in dynamic mode at 15 MPa and 45 ºC. All the samples had positive a^* values, as expected from their red colour. *Opuntia* spp. extracted by PLE in dynamic mode at 15 MPa and 45 ºC showed the highest a^* values, a^* = 81.51. It was observed positive values of b* parameter (blueness–yellowness) ranging from b^* = 13.44 of *Opuntia* spp. to the positive extracted by PLE in dynamic mode at 15 MPa and 45 ºC to b^* = 26.26 of *Opuntia* spp. extracted PLE in static mode at 15 MPa and 45 ºC. This means that all samples had a more yellow colour than blue. Chroma, which expresses the brilliance or purity of a colour, was highest in *Opuntia* spp. extracted by PLE in static mode at 15 MPa and 45 ºC. The hue angle indicates the tonality, and all samples present a red tonality ($0.16<h^o<0.37$). Moreover, it is possible to conclude that some of the colour parameters, such as L^*, a^* and C^*, decreased with increasing of betacyanin content. In contrast, the values of parameters b^* and h^* increased with increasing of betacyanin content.

Red beet pigment has been extensively commercialized as a food colorant. No. 3600 and E-162 are the commercial codes for red beet pigment in U.S.A. and Europe, respectively. According to the results, the O*puntia* spp. extracts obtained were different from commercial red beet, they present a red colour, more yellowness than blueness, but less purple than the commercial form.

3.1.1 Microencapsulation of betalain-rich *Opuntia* spp. extract

In this work, Supercritical Fluid Technology was used to develop lipidic particles of betalain-rich extract to be further incorporated in food matrices. In order to achieve that, *Opuntia* spp. juice by-products (fruit peels and seeds) were extracted conventionally with a mixture of EtOH:H_2O (50:50, v/v). This conventional extract was encapsulated by PGSS® technique into Glyceryl monoostearate, using a surfactant (Polyglyceryl-3 Polyricinoleate) and water. 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. Therefore, different process conditions, namely pressure, temperature and equilibrium time were tested in order to model the encapsulation of betacyanins *via* Response Surface Methodology, following a Central Composite Rotatable Design. The experimental conditions of encapsulation using PGSS® are present in Table 2.2.

3.1.2 *Opuntia* spp. extract characterization

The *Opuntia* spp. extract were analysed in terms of betacyanin content and antioxidant activity (Oxygen Radical Absorbance Capacity (ORAC), Hydroxyl Radical Adverting Capacity

(HORAC) and Hydroxyl Radical Scavenging Capacity (HOSC)). The betacyanin content was found to be 332.9 ± 0.1 mg/100 g of dried extract and the ORAC, HORAC and HOSC values were 149.5 ± 12.7, 74.9 ± 7.2 and 119.0 ± 14.4 (μmol/g), respectively. In Table 3.3., the colour parameters of the *Opuntia* spp. dried extract and the commercial red beet pigment are presented. The colour parameters of the *Opuntia* spp. dried extract were measured using CIELab method.

Table 3.3. Colour parameters of commercial red beet pigment and the *Opuntia* spp. dried extract.

Variable, factors, unit	**Colour parameters**					**Colour conversion to RGB**
	*L**	a*	b*	h°	C*	
***Opuntia* spp. dried extract**	28.12 ± 0.28	26.62 ± 0.52	-2.22 ± 0.07	355.70 ± 0.00	26.71 ± 0.52	
E-162 (Cai and Corke, 2000)	51.54	19.64	0.71	2.00	n.i.	

n.i.: not indicated

The commercial red beet pigment presents a higher *L** value than the *Opuntia* spp. dried extract (*L**=51.54), presenting a lighter colour. The a* value of the *Opuntia* spp. dried extract is higher than the red beet pigment (a*=26.62 and a*=19.64, respectively), indicating the red colour. The b* value of red beet pigment is 0.71 and the *Opuntia* spp. dried extract is -2.22. This means that the red beet pigment has a more yellow colour and the *Opuntia* spp. dried extract a bluer colour. The red beet pigment has a lower h^o (2.00), very similar to red colour. The h^o value of the *Opuntia* spp. dried extract is 355.70, indicating a slightly purple shade of red. This results, suggested that the produced *Opuntia* spp. dried extract is similar to the commercial red beet powder.

3.1.3 Modelling of *Opuntia* spp. extract encapsulation through PGSS®

To find the optimum conditions for encapsulation of betacyanins, the experimental design as a function of the selected main factors has to be determined. As shown in Table 2.2., the experimental plan was carried out as a Central Composite Rotatable Design (CCRD) consisting of 17 experiments. For three variables (n = 3) and five levels (-α (-1.68) low (−), medium (0) and high (+) and +α (+1.68)), the total number of experiments was 17 determined by the expression: 2^n (2^3 = 8: factor points) + 2n (2 × 3 = 6: axial points, including on the axis of each design variable at a distance of 1.68 from the design center) + 3 (center points: three replications).

The effects of each factor and the interactions between factors on the various responses were calculated. Table 3.4. shows the linear and quadratic effects of each variable and of their

interactions on the betacyanin content, ORAC, HORAC, HOSC, colour parameters and yield of collected particles, during the encapsulation process.

Table 3.4. 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 indicates that higher P and T values, within the tested range, correspond to a lower encapsulation of betacyanins. The t has demonstrated to have lower effect on the encapsulation of betacyanins. The positive quadratic effect of t indicates that the experimental results on betacyanin encapsulation can be fitted to a four-dimensional concave surface (Figure 3.4.). All the interactions between factors for the betacyanin content are important. As the P and T values increases, the betacyanin encapsulation decreases. When the P and t increases the betacyanin encapsulation increases. As T and t increases, the encapsulation of betacyanins decreases.

Concerning the ORAC values, significant linear negative effects of P and T were found, being in accordance with 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 indicate that ORAC can be described by a four dimensional concave surface (Figure 3.4.). When the P and t increases the ORAC increases, and as T and t increases the ORAC values decreases.

Regarding the HOSC values, significant linear negative effect of P and T were found, in accordance with the observations for the ORAC values. As P and T increased, the HOSC values decreased. Also, the significant quadratic positive effects of P and t indicate that ORAC can be

described by a four dimensional concave surface (Figure 3.4.). When the P and t increases, the HOSC values increased, as well.

(a) (b)

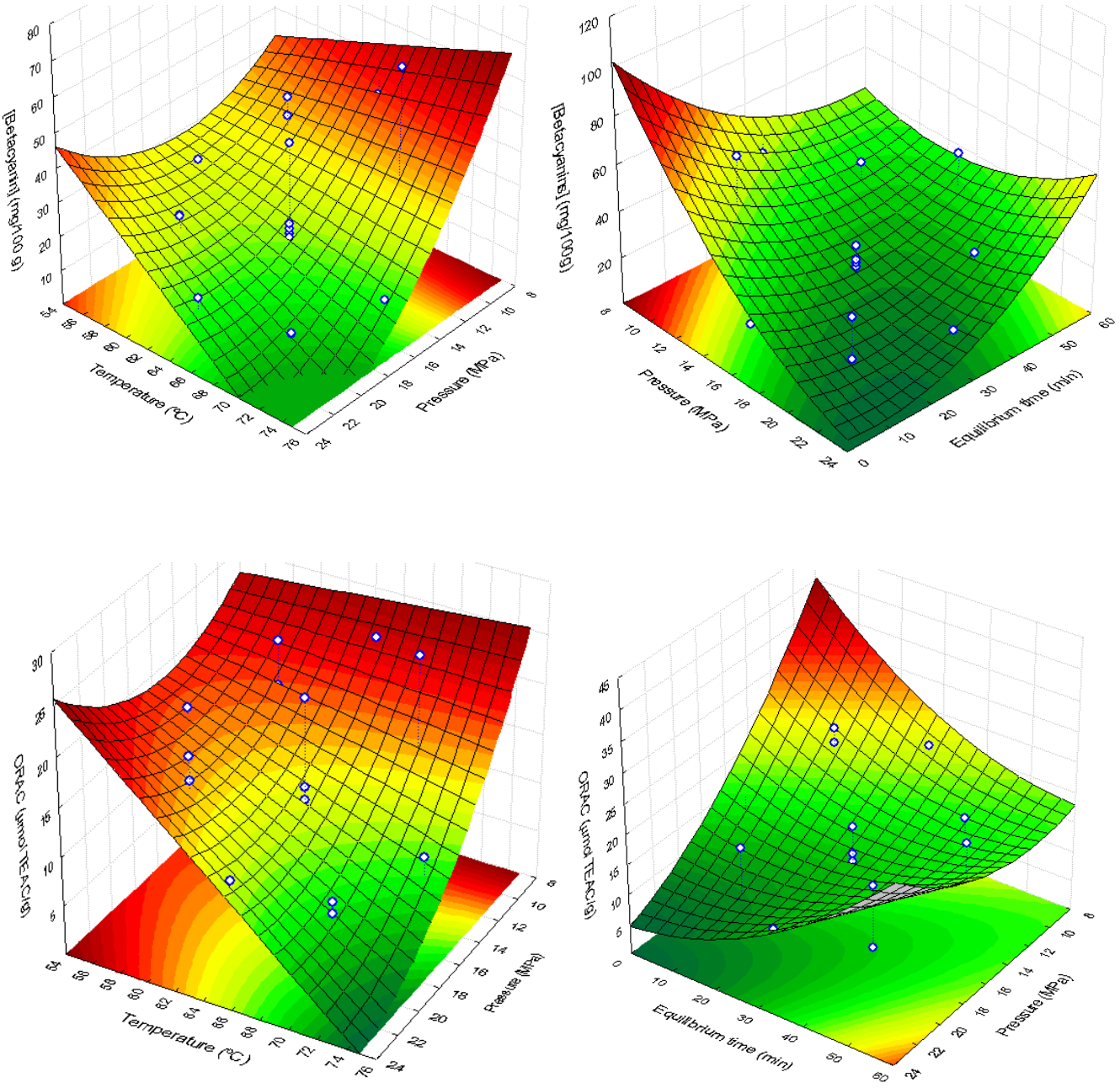

Figure 3.4. Response surface fitted to the betacyanin content and ORAC as a function of (a) temperature and pressure and (b) of equilibrium time and pressure.

(a) (b)

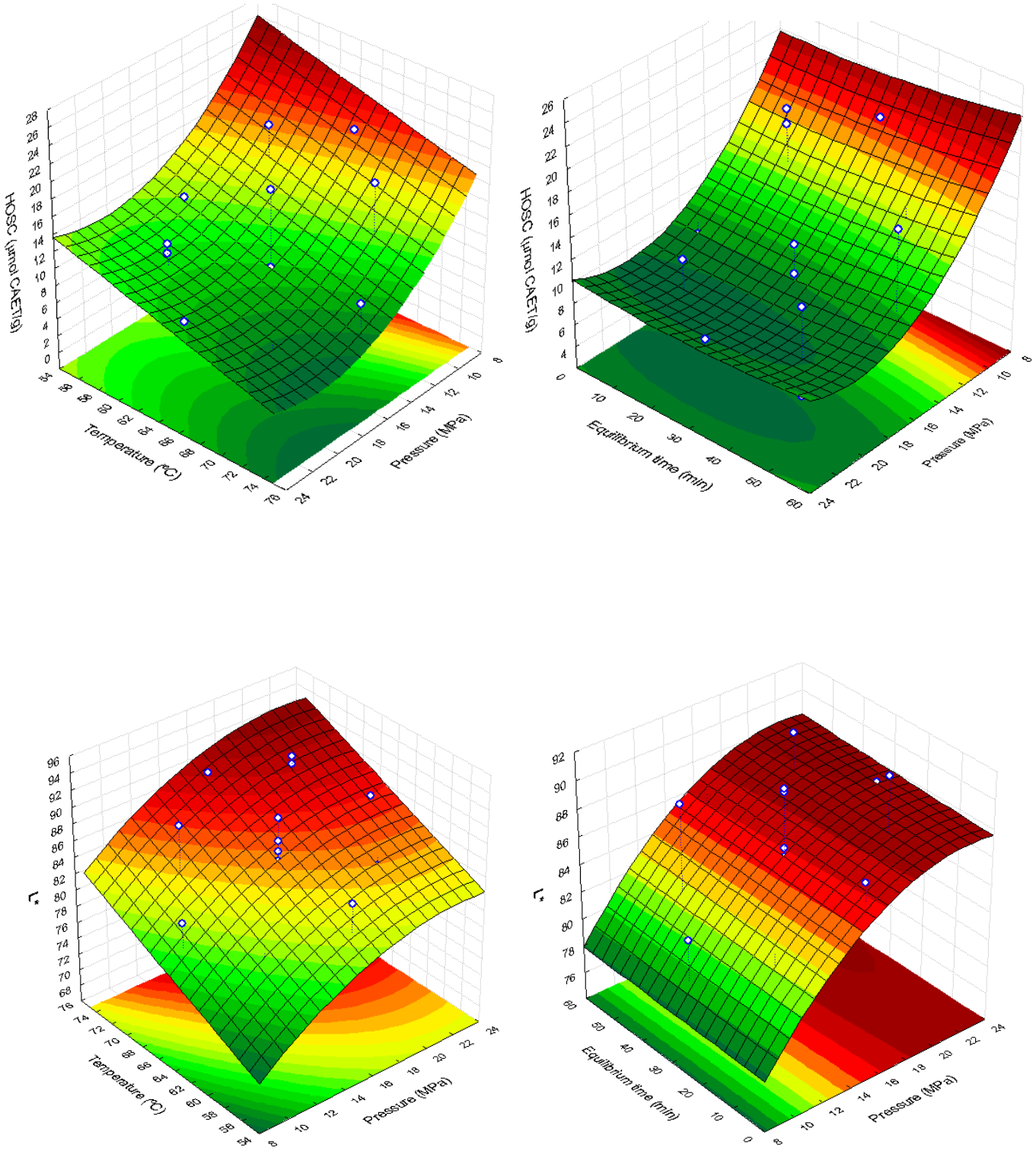

Figure 3.5. Response surface fitted to the HOSC and L^* value as a function of (a) temperature and pressure and (b) of equilibrium time and pressure.

Finally, for the results of L^*, the same trend was verified as with the ORAC and HOSC values. As P and T increased, the L^* values increased (lighter particles are obtained). Also, the significant quadratic positive effect of P and t indicates that the L^* value can be described by a four dimensional convex surface (Figure 3.5.). When the P and t increases, the L^* values decreased.

The response surfaces (Figure 3.4. and Figure 3.5.) fitted to betacyanin content, ORAC, HOSC and L^* value can be described by second-order polynomial models as a function of pressure, temperature and equilibrium time (Table 3.5.). 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 (Haaland, 1989). The values for both R^2 and $R_{adj}{}^2$ of these models (Table 3.5.) 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.

Table 3.5. 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
$L^* = 99.66 + 2.229P - 0.05P^2 + 0.514T + 0.0005Pt$	0.665	0.554

As stated before, 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/100 g. 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 by other authors were conducted for the encapsulation of pigments from *Opuntia* spp. Saenz *et al.* (2009) has 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 100 g of powder for maltodextrin and inulin, respectively (Saenz *et al.*, 2009). Another study of the encapsulation of a betacyanin-rich extract from *Opuntia lasiacantha* was performed using maltodextrin through spray-drying (Sánchez *et al.*, 2006). The amount of betacyanin in powder after drying was 65.7 mg of betanin per 100 g. The maximum content of the encapsulated pigment in this work was 64.4 ± 4.5 mg/100 g, being similar with other published work using other processes and carriers.

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

As shown in Table 3.6, 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. 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%).

The colour of the *Opuntia* spp. lipidic particles was measured using CIELab method. The results are presented in Table 3.6. From the results, 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 have the highest content in betacyanins but did not present the lowest L^* value (L^*=80.56). Regarding the C* and h° values, among all samples of the design, the C* value is between 7.50 and 20.69 and the h° values between 338.18 and 348.39, indicating a slightly purple shade of red.

Table 3.6. Summary of experimental results.

Experiment number	Yield of collected particles (%)	Betacyanin content (mg/100g dried extract)	*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*					RGB colour conversion
						*L**	a*	b*	h°	C*	
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	

SEM Microphotographs of the powders produced by PGSS® process for comparable conditions are shown in Figure 3.6. 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 (Gandia-Herrero *et al.*, 2010). The average size of particles was 10 μm, approximately.

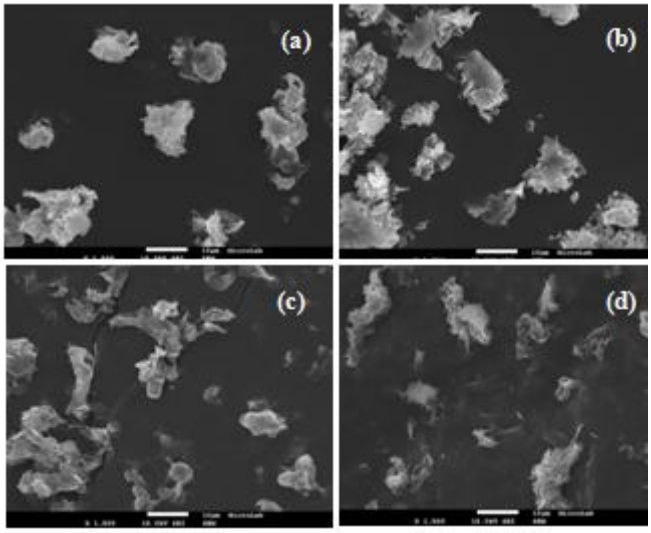

Figure 3.6. SEM pictures of powders obtained at different operating conditions and corresponding betacyanin contents (mg/100 g). (a) 12 MPa, 70 ºC and 64.4 mg/100 g; (b) 20 MPa, 70 ºC and 8.0 mg/100 g; (c) 12 MPa, 60 ºC and 41.3 mg/100 g; (d) 20 MPa, 60 ºC and 26.0 mg/100 g.

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 (Lack *et al.*, 2005; Weidner, 2009).

The melting point was evaluated for the optimum process conditions and the thermogram is presented in Figure 3.7.

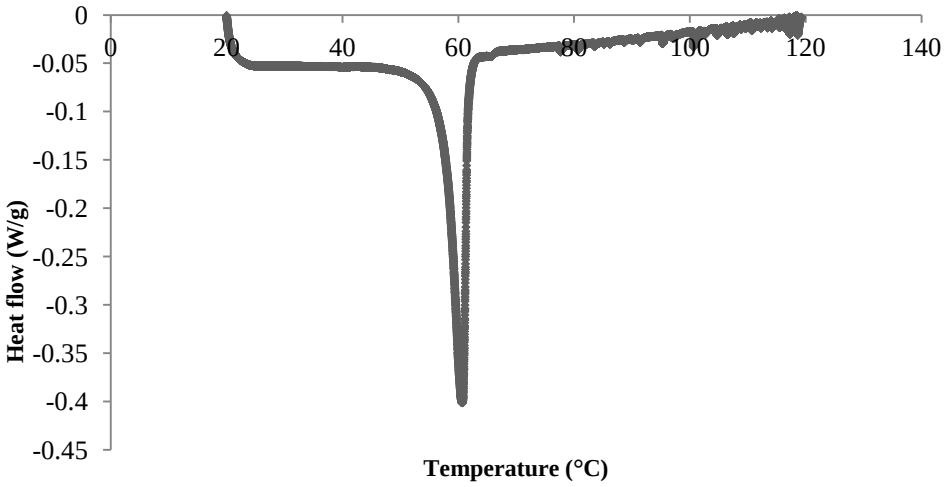

Figure 3.7. Thermogram of particles obtained for optimum conditions.

From the thermogram presented above, the melting temperature for the optimum conditions is between 57 and 65 °C.

3.1.4 Incorporation in a fat-based product

The *Opuntia* spp. extract and the particles were added to the ice cream formulation in a concentration of 1.75 mg/L. Pictures have been taken after freezing and the images are shown in Figure 3.8.

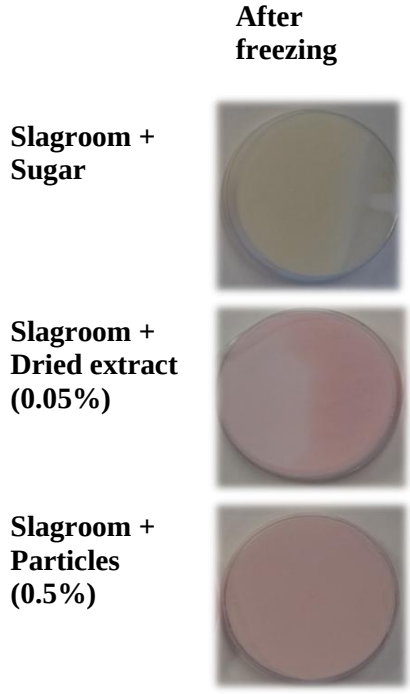

Figure 3.8. Incorporation of particles and dried extract in a food product (ice-cream).

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

4 Conclusions

The aim of this work was to develop adequate clean and mild methodologies for the isolation and stabilization of betacyanins, from cactus pear fruits (*Opuntia* spp.).

In this work, HPCDAE was shown to be a successful technology to extract betacyanins from *Opuntia* spp. fruits. Process variables, namely extraction pressure, temperature and ratio between CO_2/acidified water, had strong impact on recovery of betacyanins. The optimal HPCDAE conditions for the extraction of these pigments from *Opuntia* spp. fruits were 15 MPa, 45 ºC for the total betacyanins content of 211 ± 10 mg/100 g. Comparing this result with CE and PLE (85 ± 3, 191 ± 2 and 153 ± 5 mg/100 g, respectively), the yield of betacyanins obtained for optimal HPCDAE conditions were higher, indicating that this extraction technology may be a promising alternative to the conventional extractions methodologies applied to betacyanin-rich sources.

Furthermore, betacyanin pigments derived from *Opuntia* spp. juice by-products were successfully encapsulated in a lipidic 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. Highest betacyanin encapsulation was achieved at lower pressures. The pressure had a negative effect on betacyanin encapsulation. Lower pressures leads to an increase in the betacyanin encapsulation. This effect was more pronounced at higher temperatures and lower equilibrium time. At these conditions, Opuntia spp. particles presented 64.4 ± 4.5 mg/100 g and high antioxidant capacity. When compared with the *Opuntia* spp. dried extract, lipidic particles contributed to a better homogenization of the pink colour after incorporation in ice cream. 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 application in food industry.

5 References

Allegra, M., P. Furtmuller, W. Jantschko, M. Zederbauer, L. Tesoriere, M. Livrea, and C. Obinger, 2005, Mechanism of interaction of betanin and indicaxanthin with human myeloperoxidase and hypochlorous acid: Biochemical and Biophysical Research Communications, v. 332, p. 837-844.

Azeredo, H., 2009, Betalains: properties, sources, applications, and stability: a review: International Journal of Food Science & Technology, v. 44, p. 2365-2376.

Azmir, J., I. Zaidul, M. Rahman, K. Sharif, A. Mohamed, F. Sahena, M. Jahurul, K. Ghafoor, N. Norulaini, and A. Omar, 2013, Techniques for extraction of bioactive compounds from plant materials: A review: Journal of Food Engineering, v. 117, p. 426-436.

Boo, H., S. Hwang, C. Bae, S. Park, B. Heo, and S. Gorinstein, 2012, Extraction and characterization of some natural plant pigments: Industrial Crops and Products, v. 40, p. 129-135.

Borges, M., R. Tejera, L. Diaz, P. Esparza, and E. Ibanez, 2012, Natural dyes extraction from cochineal (*Dactylopius coccus*). New extraction methods: Food Chemistry, v. 132, p. 1855-1860.

Butera, D., L. Tesoriere, F. Di Gaudio, A. Bongiorno, M. Allegra, A. Pintaudi, R. Kohen, and M. Livrea, 2002, Antioxidant activities of Sicilian prickly pear (*Opuntia ficus indica*) fruit extracts and reducing properties of its betalains: Betanin and indicaxanthin: Journal of Agricultural and Food Chemistry, v. 50, p. 6895-6901.

Cai, Y., and H. Corke, 2000, Production and properties of spray-dried Amaranthus betacyanin pigments: Journal of Food Science, v. 65, p. 1248-1252.

Cai, Y., M. Sun, and H. Corke, 2003, Antioxidant activity of betalains from plants of the Amaranthaceae: Journal of Agricultural and Food Chemistry, v. 51, p. 2288-2294.

Cardoso-Ugarte, G.A., Sosa-Morales, M.E., Ballard, T., Liceaga, A., San Martín-González, M.F., 2014, Microwave-Assisted Extraction of betalains from red beet (*Beta vulgaris*), LWT - Food Science and Technology doi: 10.1016/j.lwt.2014.05.025.

Castellar, M., J. Obon, and J. Fernandez-Lopez, 2006, The isolation and properties of a concentrated red-purple betacyanin food colourant from *Opuntia stricta* fruits: Journal of the Science of Food and Agriculture, v. 86, p. 122-128.

Castellar, R., J. Obon, M. Alacid, and J. Fernandez-Lopez, 2003, Color properties and stability of betacyanins from Opuntia fruits: Journal of Agricultural and Food Chemistry, v. 51, p. 2772-2776.

Cavalcanti, R., T. Forster-Carneiro, M. Gomes, M. Rostagno, J. Prado, and M. Meireles, 2013, Uses and Applications of Extracts from Natural Sources, RSC Green Chemistry, p. 1-57.

de Paz, E., A. Martin, C. Duarte, and M. Cocero, 2012, Formulation of beta-carotene with poly-(epsilon-caprolactones) by PGSS process: Powder Technology, v. 217, p. 77-83.

Delgado-Vargas, F., A. Jiménez, and O. Paredes-López, 2000, Natural Pigments: Carotenoids, Anthocyanins, and Betalains — Characteristics, Biosynthesis, Processing,and Stability, Critical Reviews in Food Science and Nutrition, v. 40, p. 173-289.

Diaz-Reinoso, B., A. Moure, H. Dominguez, and J. C. Parajo, 2006, Supercritical CO2 extraction and purification of compounds with antioxidant activity: Journal of Agricultural and Food Chemistry, v. 54, p. 2441-2469.

Feliciano, R., M. Bravo, M. Pires, A. Serra, C. Duarte, L. Boas, and M. Bronze, 2009, Phenolic Content and Antioxidant Activity of Moscatel Dessert Wines from the Setubal Region in Portugal: Food Analytical Methods, v. 2, p. 149-161.

Feugang, J., P. Konarski, D. Zou, F. Stintzing, and C. Zou, 2006, Nutritional and medicinal use of Cactus pear (*Opuntia* spp.) cladodes and fruits: Frontiers in Bioscience, v. 11, p. 2574-2589.

Gacula, M., and J. Singh, 1984, Response surface designs and analysis, Statistical Methods in Food and Consumer Research, Academic Press, Orlando, p. 214–273.

Gandia-Herrero, F., and F. Garcia-Carmona, 2013, Biosynthesis of betalains: yellow and violet plant pigments: Trends in Plant Science, v. 18, p. 334-343.

Gandia-Herrero, F., M. Jimenez-Atienzar, J. Cabanes, F. Garcia-Carmona, and J. Escribano, 2010, Stabilization of the Bioactive Pigment of Opuntia Fruits through Maltodextrin Encapsulation: Journal of Agricultural and Food Chemistry, v. 58, p. 10646-10652.

Gil-Chavez, G., J. Villa, J. Ayala-Zavala, J. Heredia, D. Sepulveda, E. Yahia, and G. Gonzalez-Aguilar, 2013, Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food Ingredients: An Overview: Comprehensive Reviews in Food Science and Food Safety, v. 12, p. 5-23.

Guzman-Maldonado, S., A. Morales-Montelongo, C. Mondragon-Jacobo, G. Herrera-Hernandez, F. Guevara-Lara, and R. Reynoso-Camacho, 2010, Physicochemical, Nutritional, and Functional Characterization of Fruits Xoconostle (*Opuntia matudae*) Pears from Central-Mexico Region: Journal of Food Science, v. 75, p. C485-C492.

Haaland, P., 1989, Experimental Design in Biotechnology, New York, USA, Marcel Dekker.

Herrero, M., M. Castro-Puyana, J. Mendiola, and E. Ibanez, 2013, Compressed fluids for the extraction of bioactive compounds: Trac-Trends in Analytical Chemistry, v. 43, p. 67-83.

Herrero, M., A. Cifuentes, and E. Ibanez, 2006, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review: Food Chemistry, v. 98, p. 136-148.

Hu, W., L. Zhou, Z. Xu, Y. Zhang, and X. Liao, 2013, Enzyme Inactivation in Food Processing using High Pressure Carbon Dioxide Technology: Critical Reviews in Food Science and Nutrition, v. 53, p. 145-161.

Huang, D., B. Ou, M. Hampsch-Woodill, J. Flanagan, and R. Prior, 2002, High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format: Journal of Agricultural and Food Chemistry, v. 50, p. 4437-4444.

Huang, H., C. Hsu, B. Yang, and C. Wang, 2013, Advances in the extraction of natural ingredients by high pressure extraction technology: Trends in Food Science & Technology, v. 33, p. 54-62.

Emilia Janiszewska, 2014, Microencapsulated beetroot juice as a potential source of betalain, Powder Technology, doi: 10.1016/j.powtec.2014.05.032.

Jimenez-Aguilar, D., A. Ortega-Regules, J. Lozada-Ramirez, M. Perez-Perez, E. Vernon-Carter, and J. Welti-Chanes, 2011, Color and chemical stability of spray-dried blueberry extract using mesquite gum as wall material: Journal of Food Composition and Analysis, v. 24, p. 889-894.

Kandansamy, K., and P. Somasundaram, 2012, Microencapsulation of Colors by Spray Drying – A Review: International Journal of Food Engineering. v. 8, p. 1556-3758.

Khan, M., P. Harsha, P. Giridhar, and G. Ravishankar, 2012, Pigment identification, nutritional composition, bioactivity, and in vitro cancer cell cytotoxicity of *Rivina humilis L.* berries, potential source of betalains: Lwt-Food Science and Technology, v. 47, p. 315-323.

Lack, E., E. Weidner, Z. Knez, S. Gruner, B. Weinreich, and H. Seidlitz, 2005, Particle generation with supercritical CO_2. In Proceedings of the 1st Vienna International Conference: Micro- and Nano-Technology, Vienna, Austria.

Liu, X., Y. Gao, X. Peng, B. Yang, H. Xu, and H. Zhao, 2008, Inactivation of peroxidase and polyphenol oxidase in red beet (*Beta vulgaris L.*) extract with high pressure carbon dioxide: Innovative Food Science & Emerging Technologies, v. 9, p. 24-31.

Liu, X., Y. Gao, H. Xu, Q. Hao, G. Liu, and Q. Wang, 2010, Inactivation of peroxidase and polyphenol oxidase in red beet (*Beta vulgaris L.*) extract with continuous high pressure carbon dioxide: Food Chemistry, v. 119, p. 108-113.

Maran, J., S. Manikandan, and V. Mekala, 2013, Modeling and optimization of betalain extraction from *Opuntia ficus-indica* using Box-Behnken design with desirability function: Industrial Crops and Products, v. 49, p. 304-311.

Maran, J. P., and S. Manikandan, 2012, Response surface modeling and optimization of process parameters for aqueous extraction of pigments from prickly pear (*Opuntia ficus-indica*) fruit: Dyes and Pigments, v. 95, p. 465-472.

Moore, J., J. Yin, and L. Yu, 2006, Novel fluorometric assay for hydroxyl radical scavenging capacity (HOSC) estimation: Journal of Agricultural and Food Chemistry, v. 54, p. 617-626.

Moreno, D. A., C. García-Viguera, J. I. Gil, and A. Gil-Izquierdo, 2008, Betalains in the era of global agri-food science, technology and nutritional health: Phytochemistry Reviews, v. 7, p. 261-280.

Mustafa, A., and C. Turner, 2011, Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review: Analytica Chimica Acta, v. 703, p. 8-18.

Nemzer, B., Z. Pietrzkowski, A. Sporna, P. Stalica, W. Thresher, T. Michalowski, and S. Wybraniec, 2011, Betalainic and nutritional profiles of pigment-enriched red beet root (*Beta vulgaris L.*) dried extracts: Food Chemistry, v. 127, p. 42-53.

Obon, J. M., M. R. Castellar, M. Alacid, and J. A. Fernandez-Lopez, 2009, Production of a red-purple food colorant from *Opuntia stricta* fruits by spray drying and its application in food model systems: Journal of Food Engineering, v. 90, p. 471-479.

Osorio-Esquivel, O., Alicia-Ortiz-Moreno, V. Alvarez, L. Dorantes-Alvarez, and M. Giusti, 2011, Phenolics, betacyanins and antioxidant activity in *Opuntia joconostle* fruits: Food Research International, v. 44, p. 2160-2168.

Ou, B., M. Hampsch-Woodill, J. Flanagan, E. Deemer, R. Prior, and D. Huang, 2002, Novel Fluorometric Assay for Hydroxyl Radical Prevention Capacity Using Fluorescein as the Probe: Journal of Agricultural and Food Chemistry, v. 50, p. 2772-2777.

Pitalua, E., M. Jimenez, E. Vernon-Carter, and C. Beristain, 2010, Antioxidative activity of microcapsules with beetroot juice using gum Arabic as wall material: Food and Bioproducts Processing, v. 88, p. 253-258.

Ravichandran, K., R. Palaniraj, N. Saw, A. Gabr, A. Ahmed, D. Knorr, and I. Smetanska, 2012, Effects of different encapsulation agents and drying process on stability of betalains extract: Journal of Food Scientists & Technologists, v. 51, p. 2216-2221.

Saenz, C., S. Tapia, J. Chavez, and P. Robert, 2009, Microencapsulation by spray drying of bioactive compounds from cactus pear (*Opuntia ficus-indica*): Food Chemistry, v. 114, p. 616-622.

Sanchez-Gonzalez, N., M. Jaime-Fonseca, E. San Martin-Martinez, and L. Zepeda, 2013, Extraction, Stability, and Separation of Betalains from *Opuntia joconostle* cv. Using Response Surface Methodology: Journal of Agricultural and Food Chemistry, v. 61, p. 11995-12004.

Santos, D., and M. Meireles, 2011, Optimization of bioactive compounds extraction from jabuticaba (*Myrciaria cauliflora*) skins assisted by high pressure CO_2: Innovative Food Science & Emerging Technologies, v. 12, p. 398-406.

Serra, A., J. Poejo, A. Matias, M. Bronze, and C. Duarte, 2013, Evaluation of *Opuntia* spp. derived products as antiproliferative agents in human colon cancer cell line (HT29): Food Research International, v. 54, p. 892-901.

Shahid, M., Shahid-ul-Islam, and F. Mohammad, 2013, Recent advancements in natural dye applications: a review: Journal of Cleaner Production, v. 53, p. 310-331.

Stintzing, F., and R. Carle, 2007, Betalains - emerging prospects for food scientists: Trends in Food Science & Technology, v. 18, p. 514-525.

Stintzing, F., K. Herbach, M. Mosshammer, R. Carle, W. Yi, S. Sellappan, C. Akoh, R. Bunch, and P. Felker, 2005, Color, betalain pattern, and antioxidant properties of cactus pear (*Opuntia* spp.) clones: Journal of Agricultural and Food Chemistry, v. 53, p. 442-451.

Strack, D., T. Vogt, and W. Schliemann, 2003, Recent advances in betalain research: Phytochemistry, v. 62, p. 247-269.

Sun, H., X. Ge, Y. Lv, and A. Wang, 2012, Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed: Journal of Chromatography a, v. 1237, p. 1-23.

Sánchez, F., E. López, S. Kerstupp, R. Ibarra, and L. Scheinvar, 2006, Colorant Extraction from red prickly pear (*Opuntia lasiacantha*) for food application., Electronic Journal of Environmental, Agricultural and Food Chemistry, v. 5, p. 1330-1337.

Tesoriere, L., D. Butera, M. Allegra, M. Fazzari, and M. Livrea, 2005, Distribution of betalain pigments in red blood cells after consumption of cactus pear fruits and increased resistance of the cells to ex vivo induced oxidative hemolysis in humans: Journal of Agricultural and Food Chemistry, v. 53, p. 1266-1270.

Tiwari, B., and P. Cullen, 2013, Red Beet Biotechnology: Food and Pharmaceutical Applications, Springer Science+Business Media New York, v. 14, p. 373-391.

Vergara, C., J. Saavedra, C. Saenz, P. Garcia, and P. Robert, 2014, Microencapsulation of pulp and ultrafiltered cactus pear (*Opuntia ficus-indica*) extracts and betanin stability during storage: Food Chemistry, v. 157, p. 246-251.

Wang, L., and C. Weller, 2006, Recent advances in extraction of nutraceuticals from plants: Trends in Food Science & Technology, v. 17, p. 300-312.

Weidner, E., 2009, High pressure micronization for food applications: Journal of Supercritical Fluids, v. 47, p. 556-565.

Weisberg, S., 1985, Applied Linear Regression, New York, USA, John Wiley & Sons, p. 217–218.

Wimmer, Z., and M. Zarevucka, 2010, A Review on the Effects of Supercritical Carbon Dioxide on Enzyme Activity: International Journal of Molecular Sciences, v. 11, p. 233-253.

Xu, Z., J. Wu, Y. Zhang, X. Hu, X. Liao, and Z. Wang, 2010, Extraction of anthocyanins from red cabbage using high pressure CO_2: Bioresource Technology, v. 101, p. 7151-7157.