SUPERCRITICAL FLUID EXTRACTION, MICRO-ENCAPSULATION AND APPLICATION OF BETACYANIN-EXTRACT FROM RED PITAYA (Hylocereus polyrhizus (WEBER) BRITON & ROSE)

FARAHNAZ FATHORDOOBADY

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By

FARAHNAZ FATHORDOOBADY

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

September 2015

DEDICATION

This thesis is dedicated to

My Dear Husband

Who always supports me with his love

My Dear Daughters

and

My Parents

Who taught me to be strong and tolerant

&

Whose souls are my eternal guardian angel

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

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Chairman : Professor Dato Mohd Yazid Abd Manap, PhD
Faculty : Food Science and Technology

There is a progressive demand for natural pigments in colouring food stuff instead of synthetic colourants because of human health concern. Red pitaya (*Hylocereus polyrhizus*) is found to be a promising source of more stable natural red pigment of betacyanins, with a deep purple colour compared to red beet, commercial source of betacyanins pigment, and is also regarded as a rich source of antioxidants. Due to the undesirable properties of betacyanins from beet root and adverse environmental effects of solvent extraction which is mostly used for betacyanins recovery, the aim of present study was to investigate an appropriate method for obtaining betacyanins from *Hylocereus polyrhizus* and increasing the heat and storage stability of pigment extract from the fruit peel, a waste product of juice processing which would be considered as a natural red pigment with bioactive properties. In this regard supercritical fluid extraction (SFE) with CO_2 as solvent and EtOH/water mixture as co-solvent was used for the extraction of betacyanins. Pressure and temperature of process as the most significant parameters affecting extraction condition as well as co-solvent percentage which improves the extraction of polar bioactive materials, were chosen as supercritical fluid extraction variables. It was found that SFE method had betacyanins recovery of 86.7-92.5% compared to solvent extraction. A 2^3 factorial design was used for optimization of SFE method and investigation the effects of extraction parameters (temperature, pressure and co-solvent concentration) on response variables of yield (%), total betacyanins content (mg/100 ml), colour properties (a^*) and antioxidant activity (IC_{50}) of extracts. The linear effects of pressure, temperature and co-solvent concentration were found to be significant on all the response variables ($p<0.05$). Optimal SFE conditions were identified as 30 MPa pressure, 40ºC temperature, and 20% co-solvent for the flesh, and 25 MPa pressure, 50ºC temperature, and 15% co-solvent for the peel. The response variables at optimal point were assessed as maximum extraction yield of 3.93% and 10.90 %, total betacyanins content of 25.67 mg/100 ml and 98.65 mg/100 ml, redness (a^*) of 59.29 and 57.51, and IC_{50} of 1.2 mg/ ml and 2.1 mg/ ml for the peel and flesh, respectively. Experimental values for response variables at these optimal conditions matched well with the predicted values. The pigment profile of the peel and flesh extracts from solvent and SFE methods was detected using HPLC and ESI- LC/MS/MS technique. Betanin, phyllocactin and their C-15 respective isoforms were the major betacyanins constituents detected in all of the samples of extracts. In this study, hylocrenin and its C-15 isoform which were previously identified in *Hylocereus* genus were detected in trace amounts. In order to improve the stability of betacyanins extract of the peel of *Hylocereus polyrhizus* obtained through the optimal condition of SFE, a good source of betacyanins pigment with potent antioxidant activity, ionization gelation method of micro-encapsulation with alginate as wall material was used. Based on the equation derived from RSM-face centered composite design of experiment, the combination of 2.9% alginate (%), 161.9 mM $CaCl_2$ (mM) and 26.4% betacyanins extract (v/v) was identified as the optimal mico-encapsulation condition for high efficiency, minimum mean particle size and the best matrix uniformity of micro-encapsulated extract. To assess the potential of micro-capsules as an antioxidant carrier, the release behavior of alginate-loaded betacyanins extract in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was also investigated. The results of heat stability and storage study showed significant improvement ($p<0.05$) of half-life, and total betacyanins and antioxidant activity retention in micro-encapsulated betacyanins extract in comparison with non-capsulated extract and commercial betanin solution (control). Further study on the application of micro-encapsulated betacyanins extract in two food systems (yoghurt drink and jelly) revealed that during storage at 4 °C for 60 days, the retention of total betacyanins content and antioxidant activity in two food products with betacyanins micro-capsules was significantly higher ($p<0.05$) than in products colourd with non-capsulated betacyanins and betanin solution. Sensory attributes evaluation of prepared yoghurt drinks and jelly samples coloured with betacyanins micro-capsules represented significant higher overall acceptability ($p<0.05$) compared to two other samples. Moreover, the products containing betacyanins extract micro-capsules had faced lower colour changes ([illegible]) compared to products coloured with non-capsulated betacyanins and commercial betanin (control) during storage condition. These findings revealed that betacyanins micro-capsules from the peel of *Hylocereus polyrhizus* is an alternative natural red pigment with high potential of antioxidant activity and desired stability, and can be exploited for some food formulation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

SUPERCRITICAL BENDALIR PERAHAN, MICRO-PENGKAPSULAN DAN PEMAKAIAN BETACYANIN-EKSTRAK DARIPADA RED PITAYA (*Hylocereus polyrhizus* (WEBER) BRITON & ROSE)

Oleh

FARAHNAZ FATHORDOOBADY

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Pengerusi : Profesor Dato Mohd Yazid Abd Manap, PhD
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Terdapat permintaan yang progresif untuk pigmen semula jadi dalam pewarna barangan makanan dan bukan pewarna sintetik kerana kebimbangan kesihatan manusia. Betacyanin dari pitaya merah (*Hylocereus polyrhizus*) didapati menjadi pigmen merah semulajadi cerah dan lebih stabil dengan mendalam ungu berwarna berbanding bit merah atau Amaranth, dan juga dianggap sebagai sumber yang kaya dengan antioksidan. Antara kaedah yang berbeza pengekstrakan, pengekstrakan cecair genting lampau dengan CO_2 didapati menjadi salah satu kaedah yang paling wajar dan mesra alam dan telah digunakan dengan cekap untuk pengekstrakan bahan-bahan semula jadi dan produk tambah nilai. Dalam permohonan kajian ini kaedah CO_2 superkritikal pengekstrakan dengan campuran air / etanol sebagai co-pelarut untuk pengekstrakan betacyanins dari kulit dan daging pitaya merah didapati mempunyai pemulihan 86.7-92.5% berbanding pelarut pengekstrakan. A 2^3 reka bentuk faktorial telah digunakan untuk siasatan kesan pelbagai parameter pengekstrakan (suhu, tekanan dan kepekatan pengubahsuai) pada hasil%, jumlah kandungan betacyanin (mg / 100ml), ciri-ciri warna (*a* *) dan aktiviti antioksidan (IC_{50}) ekstrak. Kesan linear tekanan, suhu dan kepekatan bersama pelarut telah didapati signifikan pada semua respons yang berubah-ubah (p <0.05). Keadaan SFE terbaik untuk pengekstrakan betacyanin yang dikira seperti berikut: tekanan, 25 dan 30MPa, suhu, 40 dan 50 ℃; dan bersama-pelarut, 15 dan 20% untuk kulit dan daging, masing-masing. Nilai dinilai seperti berikut: kadar pengeluaran maksimum 3.93 ± 0.8 dan 10.90 ± 0.07%; jumlah kandungan betacyanin daripada 25,67 ± 0.19 dan 98.65 ± 0.31 mg / 100ml; kemerahan (*a* *) daripada 59.29 ± 0.16 dan 57.51 ± 0.33; dan IC_{50} sebanyak 1.2 ± 0.18 dan 2.1 ± 0.15 (mg / ml) untuk kulit dan daging, masing-masing. Nilai eksperimen untuk pembolehubah sambutan pada keadaan optimum dipadankan dengan baik dengan nilai-nilai yang diramalkan. Profil pigmen kulit dan daging cabutan daripada kaedah pengekstrakan cecair pelarut dan genting lampau telah dikesan menggunakan HPLC dan ESI- LC / MS / MS kaedah analisis. Betanin, phyllocactin dan C-15 isoforms masing-masing ialah betacyanins juzuk utama dikesan di semua sampls ekstrak. Dalam kajian ini, hylocrenin dan isoform C-15 yang yang sebelum ini dikenal pasti dalam genus Hylocereus dikesan dalam jumlah surih. Dalam usaha untuk meningkatkan betacyanin kestabilan,

kaedah penggelan pengionan pengkapsulan dengan alginat sebagai bahan dinding diamalkan untuk pemikrokapsulan ekstrak pigmen optimum diperoleh melalui pengekstrakan cecair genting lampau daripada betacyanins dari kulit daripada Hylocereus polyrhizus. Berdasarkan persamaan yang diperolehi dari RSM-Face Berpusat Design Komposit eksperimen, gabungan sebanyak 2.9% alginat (%), 161.9 mM $CaCl_2$ (mM) dan 26.4% Be-ekstrak (v/v) adalah keadaan yang optimum untuk kecekapan yang lebih baik pengkapsulan serta minimum saiz zarah min dan matriks keseragaman terbaik ekstrak yang terkandung. Kajian tingkah laku pelepasan alginat-dimuatkan betacyanin ekstrak menunjukkan kestabilan wajar dalam simulasi cecair gastrik (SGF) dan cecair usus simulasi (SIF). Kestabilan haba dan rak-hidup terkandung Be-ekstrak telah juga meningkat dengan ketara (p <0.05) berbanding dengan ekstrak bukan capsulated dan penyelesaian betanin komersial. Kajian lanjut atas permohonan terkandung Be-ekstrak dalam dua sistem makanan (minuman yogurt dan agar-agar) mendedahkan bahawa produk berwarna dengan Be-mikro mempunyai nilai-nilai yang lebih rendah daripada perubahan warna (û *) dalam perbandingan dengan produk berpigmen oleh betacyanin bukan capsulated dan betanin komersial (kawalan) pada 60 hari penyimpanan pada 4 ℃. Aktiviti antioksidan dua produk makanan dengan Be-mikro dikekalkan lebih daripada 2 kali ganda berbanding produk dengan dua pigmen semasa kajian penyimpanan. Sifat-sifat penilaian deria disediakan minuman yogurt dan sampel jeli berwarna dengan mikro betacyanin diwakili penerimaan keseluruhan yang lebih tinggi yang signifikan (p <0.05) berbanding dengan sampel kawalan. Penemuan ini menunjukkan bahawa Be-mikro dari kulit daripada *Hylocereus polyrhizus* adalah pigmen merah semula jadi alternatif yang berpotensi tinggi aktiviti antioksidan dan kestabilan dikehendaki dan boleh dieksploitasi untuk formulasi makanan.

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I certify that a Thesis Examination Committee has met on 29 September 2015 to conduct the final examination of Farahnaz Fathordoobady on her thesis entitled "Supercritical Fluid Extraction, Micro-Encapsulation and Application of Betacyanin-Extract from Red Pitaya (*Hylocereus polyrhizus* (Weber) Britton & Rose)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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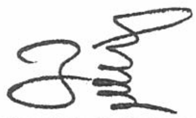

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variances
AOAC	Association of Official Analytical Chemists
AW	Active Water
Be	Betacyanins
CIE	International Commission on Illumination
CRD	Complete Randomized Design
DAA	Days After flower Anthesis
DF	Degree of Freedom
DOPA	Dihydroxyphenylalanine
DPPH	1-diphenyl-2-picrylhydrazyl
EDTA	Ethylene Diamine Tetraacetic acid
EE	Encapsulation Efficiency
ESI	Electro-Spray Ionization
EU	European Union
E/W	Ethanol/Water
FESEM	Field Emission Scanning Electron Microscopy
FDA	Food and Drug Administration
FRAP	Ferric Reducing Antioxidant Power
GA	Gallic Acid
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibition Concentration for 50% decrease
LC/MS	Liquid Chromatography/ Mass Spectrometry
MPa	Mega Pascal
NMR	Nuclear Magnetic Resonance

PDA	Photo Diode Array
RC	Regression Coefficient
RP	Reverse Phase
RSM- FCCD	Response Surface Methodology- Face Centered Composite Design
SC-CO_2	Supercritical Carbon Dioxide
SFE	Supercritical Fluid Extraction
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
TPC	Total Phenol Content
TFA	Trifluoroacetic acid
TPTZ	Tripyridy-S-Triazine
UV-VIS	Ultra Violet Visible

CHAPTER 1

INTRODUCTION

Colour plays an important role in our life. One of the main features of food quality evaluation is colour. By employing appealing colourants, users can enjoy the taste and flavour of the food they eat. Colour can also be a determining factor in considering a food as safe, with satisfied sensorial specifications. Natural pigments derived by ecofriendly methods are extremely popular. The demand can be fulfilled by providing more healthy ways of colouring foodstuff and investigating novel green methods of pigment production. The most distinguished plant pigments consist of carotenoids, chlorophylls, anthocyanins and betalains. Whereas the first two categories are deposited in particular plastids in plant cells, the latter groups are located in vacuoles. Among these natural pigments, betalains recently attracted researches attention due to the desirable properties. Up to present, about 75 betalains have been structurally identified from 17 out of 34 plant families under the order Caryophyllales (Khan and Giridhal, 2015). These components are nitrogen containing pigments with a core structure known as betalamic acid. Condensation of betalamic acid with its glucosyl derivatives of cyclo-DOPA, and its derivatives of amino acids results in formation of two types of betalains: violet betacyanins and yellow betaxanthins (Cai *et al.*, 2005; Bakar *et al.*, 2011). Betacyanins pigment as a significant component of betalains, is associated with the most abundant red colour exhibited by fruits, flowers and other parts of plants, and is found in about 12 plant families of the Chenopodiineae sub-order. *Beta, Amaranthus* and *Hylocereus* genera are the most known sources of these components. Up to present, betacyanins from red beet (*Beta vulgaris*) have been broadly studied and approved as colour additive in the United State (No. 1600) and in the European Union (E-162) (Stintzing and Carle, 2007).

Background of study: Betacyanins pigment as a colour agent is a potent candidate for colouring low acid foodstuffs such as dairy products and beverages. As anthocyanins lose their pictorial power and shade at pH from 3 to 7, betacyanins are more stable in this condition. Red-beet is the only source of commercial betacyanins pigment known in European Union as E-162 (Moßhammer *et al.*, 2005). However, owing to nitrate accumulation which may lead to the formation of nitrosamines, micro-organisms carry-over and earthy smell caused by pyrazine and geosmin and derivatives in red beet, searching for new betacyanins containing plants seems necessary.

Recently, fruits from *Cactaceae* family have been considered as rich sources of betacyanins which have the potential to be natural food colouring agents (Lim *et al.*, 2011). Among them, red-fleshed pitaya fruit (*Hylocereus polyrhizus*) native to Thailand, Vietnam, Taiwan, South of America and some other parts of the world is becoming popular in Malaysia due to its unique appearance, appealing red-purple colour and great nutritive value and bioactive specifics. The fruit farm size is increasing substantially because of the high demand for fresh consumption and processing (Nurul & Asmash, 2014; Lim et al., 2010). Red dragon fruits harvested close to full peel colour development keep their visual acceptance and marketing quality stored for at least 3 weeks at 6 ℃, 2 weeks at 14 ℃ and 1 week at 20 ℃ (Nerd *et al.*, 1999). Red pitaya peel which accounts for around 33% of whole fruit weight can be considered as a source of betacyanins pigment. Pitaya peels are often discarded during processing, especially in the beverage production industries. Harivaindaram *et al*. (2008) and Ding *et al*. (2009) had suggested red pitaya peel potential as a natural colourant and thickening agent or as a moisturizer in cosmetic products. It contained considerable amount of pectin, betacyanins pigment, phenolic compounds and total dietary fibre. Hence, pitaya peel could be utilized as a good source of fibre, pectin and natural colourant. (Bakar *et al.*, 2011).

Extraction of betacyanins from the plant sources is accompanied with releasing them from the vacuolar cells where they are found. Extraction is accomplished by applying organic solvents like methanol, ethanol, acetone and ethylene glycol or mixture of solvents followed by centrifuging, filtering and vacuum concentration. There are methods of extraction of betacyanins in laboratory scale such as fermentation with yeast, gel filtration chromatography, diffusion extraction, reverse osmosis, ultrafiltration and aqueous two phase extraction (Thakur and Gupta, 2006; Chethana *et al.*, 2007). However, the problem with these methods is that they provide poor yields and are not worth in commercial scale. The only advantage of these methods is that they were just effective on recovering betacyanins from red beet tissue when compared to conventional methods. On the other hand, there is an increasing attempt to limit the application of organic solvents in food industry since the residue of organic solvents is considered as a food safety issue.

One significant alternative to conventional methods is supercritical fluid extraction (SFE), a green and natural way of natural matter extraction. SFE has received improved attention as an alternative to conventional isolation methods since it is faster, simpler, more efficient and it avoids applying of huge amounts of organic solvents, which are often both very costly and potentially harmful (Nisha *et al.*, 2012). Low extraction temperature, continuous flow of fresh fluid, faster mass transfer and high selectivity, capability of handling the solvation power of solvent by altering pressure and/or temperature, are the advantages of this method (Pasquali *et al.*, 2008; Sahena *et al.*, 2009). While SFE method was efficiently applied for extraction of bioactive anthocyanins compounds (Vatai *et al.*, 2009; Ghafoor *et al.*, 2010; Xu *et al.*, 2010; Santos and Meireles, 2011; Santos *et al.*, 2012), no study has been recorded regarding supercritical fluid extraction of betacyanins yet.

When betalains are employed as food colouring agent, the stability of colour becomes a main concern. Many features affect the pigment stability. Water activity, a crucial factor governing the pigment liability to cleavage of aldimine –bond, can be considered one of these factors (Herbach *et al.*, 2006b). Atmosphere also has an effect on degradation of betalains. This pigment reacts with molecular oxygen. The other significant factor is light. Betalain colour strength was found to be diminished by light exposure (Azeredo *et al.*, 2009) . The most essential factor that may affect stability of betalain during processing and storage is temperature. Betalains are generally recognized to be heat-labile and lose their stability at high temperatures (Herbach *et al.*, 2006b), and the rate of degradation is accelerated by applying elevated temperature in longer period of heating. Some actions of metal such as iron, tin, copper, and aluminium also have accelerating influence on betalains degradation (Herbach *et al*., 2006b; Azeredo *et al*., 2009) .

Among different methods of increasing the stability of active materials, micro-encapsulation is found to be a significant substituting approach in improving the stability of labile bioactive compounds such as betacyanins. Spray drying was the most applied technique of micro-encapsulation for betacyanins encapsulation (Cai and Corke, 2000; Azeredo *et al.*, 2007; Saénz *et al.*, 2009; Pitalua *et al.*, 2010; Ravichandran *et al.*, 2012; Gandía-Herrero *et al.*, 2013; Vergara *et al.*, 2014). Due to heat sensibility of betacyanin-type pigments, micro-encapsulation through spray drying resulted to relative loss of these compounds. Alternatively, ionization gelation method with alginate as wall material has been well-known for its mild condition of encapsulation which makes it suitable for heat sensitive compounds such as betacyanins. Sodium alginate, a linear hetero-polysaccharide of *D*-mannuronic and *L*-guluronic acids drawn from different species of algae, is recognized as the non-toxic, biodegradable, naturally occurring anionic polysaccharides with high biological safety. It can be cross-linked by cations such as Ca^{2+} to make a polyelectrolyte complex coating of insoluble calcium alginate network (Chuah *et al.*, 2009). Alginate microspheres have found pronounced potential in encapsulation of valuable products such as drugs (Sezer and Akbuga, 1999), proteins and enzymes (DeGroot and Neufeld, 2001; Gombotz and Wee, 2012), cells (Smidsrød, 1990; Choi *et al.*, 2007; Zhang and He, 2009), probiotics (Dong *et al.*, 2013), and flavours (de Roos, 2003). Alginate is also used as a thickener and texture improver in syrups, sauces and yoghurt. It can be applied as stabilizers in ice creams producing a smoother texture. Instant dessert jellies can be made from calcium alginate mixtures by simply mixing powders with water. All of the mentioned features make alginate a suitable material for encapsulating of bioactive betacyanins food colourant.

Problem statement: This study purposes to find solutions for the following existing problems regarding extraction and application of natural red betacyanins pigment:

1. Technological and sensorial disadvantages, nitrate accumulation and microbial carry-over risk of betacyanins extraction from beet root, the only natural commercial source of betanin-pigment.
2. Undesirable environmental impacts of solvent extraction of betacyanins.
3. Considerable amounts of red-pitaya peels as juice production wastes.
4. Scarce information regarding increasing stability of bioactive betacyanin pigment through mild micro-encapsulation methods.

Objectives of study: Based on the existing problems, the present study aims at the following main objectives in order to investigate the proper and safe method for obtaining betacyanins from the peel and flesh of *Hylocereus polyrhizus* and micro-encapsulation of betacyanins from the peel of fruit, a waste product of juice processing which can be used as a natural red pigment with bioactive properties:

1. To compare method of supercritical fluid extraction of betacyanins from *Hylocereus polyrhizus* and conventional solvent extraction regarding characteristics and bioactive properties of pigment extract
2. To optimize the yield, betacyanins recovery, colour properties and antioxidant activity of betacyanins pigment extract through supercritical fluid extraction with ethanol-water mixture as co-solvent using factorial design of experiment

3. To optimize the efficiency of micro-encapsulation and mean particle size and distribution of betacyanins pigment extract loaded to alginate microcapsules using response surface methodology

4. To identify the release behavior, shelf-life and heat-stability of micro-encapsulated betacyanins pigment extract

5. To determine the shelf-life and sensory properties of two food system coloured with micro-encapsulated betacyanins pigment

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