AN ABSTRACT OF THE THESIS OF

Wenjie Wang for the degree of Master of Science in Food Science and Technology presented on March 9, 2016.

Title: Investigation of Optimal Extraction Conditions and Microencapsulation Technique for Stabilizing Anthocyanins and Polyphenols from Fruit Materials

Abstract approved:

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The objectives of this research were to investigate the optimal conditions for anthocyanins extraction from different anthocyanin rich fruit and to develop microencapsulation formulation for improving stability of anthocyanin extracts. In the extraction optimization study, two extraction methods, "conventional solvent extraction (CE)" and "ultrasound-assisted extraction (UE)" for three different anthocyanin-rich fruit, blueberries, cherries and red pear peels, were investigated. For each extraction method, 3 extraction factors and 3 levels for each factor were evaluated: solvent type (methanol, ethanol and acetone), solvent concentration (60%, 70% and 80%), as well as extraction temperature (50, 60 and 70 ^{o}C) for CE or extraction time (20, 40 and 60 min) for UE. A L_9 (3x3) Taguchi design was employed to determine the most significant two factors, and then a completely randomized two factorial (2x2) design was applied to decide the optimal level for each factor ($P< 0.05$).

The extraction optimization was determined based on the high retention of total phenolic content (TPC), total monomeric anthocyanin (TMA), and radical scavenging capacity (DPPH assay) in extracts. Percent polymeric color (PPC) and individual anthocyanin distribution (HPLC analysis) of the extracts were also monitored to identify possible anthocyanin degradation during extraction. The optimum extraction conditions were identified as: 60% methanol, 50 ℃, for 1 hour using CE or 70% methanol, 30 ℃, for 20 min using UE for blueberries; 60% ethanol, 70 ℃, for 1 hour using CE or 80% ethanol, 30 ℃, for 20 min using UE for cherries; 60% methanol, 50 ℃, for 1 hour using CE or 60% ethanol, 30 ℃, for 60 min using UE for red pear peels. HPLC analysis identified different anthocyanin species from the three fruit extracts. Anthocyanin species, including delphinidin, cyanidin, petunidin, pelargonidin, peonidin, or malvidin with different sugar moiety in fruit extracts were altered by different extraction conditions. Therefore, different conditions for both CE and UE methods should be implemented for specific fruit aiming different anthocyanin compositions.

To prevent the environmental attacks on the stability of anthocyanin extracts during processing or storage, ionic gelation induced microencapsulation was applied to stabilize blueberry anthocyanin extracts (BB ACN) by forming the capsules between a cationic polymer, chitosan (CH), and two different anionic crosslinking agents: 1) sodium tripolyphosphate (TPP), a conventional inorganic agent, and 2) cellulose nanocrystals (CNC) as a newly found organic agent. A 3-step study was implemented. Firstly, the effect of titration direction of different crosslinking agents was evaluated for each formulation group on the yield of microcapsules (YOM),

TMA recovery, and particle characteristics. Secondly, the role of anionic crosslinking agent in encapsulation was investigated, and the encapsulation formulation was optimized to obtain BB ACN microcapsules with higher YOM and TMA recovery. TPC and DPPH for the free phenolic compounds remained in supernatants after collecting anthocyanin microcapsules were also measured, in which the lower TPC and DPPH observed in the supernatants indicated the better encapsulation performance. Thirdly, the effect of the amount of loaded BB ACN (0.41-26.06 cyaniding-3-glucoside mg/mL) on encapsulation efficiency was studied. In addition, ACN distribution in the obtained microcapsules (TMA attached on surface, bound with matrix, or freely existed in core) was also measured for evaluating the stability of formed microcapsules.

Our results showed that the titration direction of the crosslinking agent had no significant effect on TMA recovery as long as the same encapsulation formulation was used. BB-CH-CNC microcapsules exhibited significantly ($p<0.05$) higher encapsulation efficiency (up to 94%) than BB-CH-TPP. High YOM and TMA recovery was found when the concentration of anionic crosslinking agent (both CNC and TPP) was up to 1.0% (w/v) (mass ratio of chitosan and crosslinking agent = 1:10). Light microscope images clearly showed BB ACN entrapped in microcapsules in use of BB-CH-CNC formulation. ACN distribution in the microcapsules varied depending on the amount of loaded BB ACN. In the BB-CH-TPP microcapsules, 95% of TMA were entrapped in the matrix of wall materials, and the greater the amount of BB ACN loaded, the less the TMA found in the cores. However, BB-CH-CNC microcapsules had more freely available TMA in the cores (up to 48%) with

less bound in the matrix and on the surfaces with increasing loading of BB ACN. This study provided new insights on the use of chitosan based microencapsulation technique for stabilizing BB ACN, in which CNC as an anionic crosslinking agent was more effective to produce rigid and stable microcapsules with high encapsulation efficiency, compared to TPP.

Investigation of Optimal Extraction Conditions and Microencapsulation Technique for Stabilizing Anthocyanins and Polyphenols from Fruit Materials

by

Wenjie Wang

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented March 9, 2016

Commencement June 2016

Master of Science thesis of Wenjie Wang presented on March. 9, 2016.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Wenjie Wang, Author

ACKNOWLEDGEMENTS

When looking back at the last two and half years of M.S. study in the Department of Food Science and Technology, at Oregon State University, I am so grateful for having this experience as one of the most fantastic adventures in my life. During this period of time, I have grown more mature as an individual, developed many new skills as a student researcher, and also became more confident.

For all these, firstly I would like to dedicate my heartfelt thanks to my major adviser, Dr. Yanyun Zhao, for her patient guidance throughout my M.S. study. She always listened to me and gave timely and reliable feedbacks and suggestions when I struggled in some dilemma whether in the research or personal issues, which meant a lot for my professional and personal development.

I am also very thankful to Dr. Elizabeth Tomasino and Dr. Bob McGorrin for being my committee members and sharing their insights about how to well design the experiments and encourage me to think deeply in my study. I also appreciate Dr. Cherian for serving as my graduate council representative and providing valuable comments on my research.

I am very grateful to all the office staff in Wiegand Hall, Ms. Linda Dunn, Ms. Linda Hoyser, Ms. Christina Hull and Ms. Debby Yacas for their patient assistance in filing various forms, purchasing lab supplies, and many other needs. They are always nice to us, timely informed us on important network opportunities and celebrated festival happiness with us every year. I would also like to recognize Mr. Jeff Clawson

and Mr. Brian Yorgey for their generously helps on taking care of our equipment needs in the pilot plant and assist my TA works.

Many thanks give to Mr. Dan Smith and Ms. Jessica Just for the warmest supports on lab instruments and many common trivia.

I started my graduate study here as a freshly graduated undergraduate, and faced many challenges at the being of graduate study. But I am so fortunate to have the best lab mates who are always back me up whenever I faced challenges and difficulties. Here, I would like to acknowledge Dr. Jooyeoun Jung, Dr. George Cavender, Virginia and Zilong for providing every support as needed in research and life like a family. We really have many good times in our lab. Also, I want to appreciate all the friendships in United States, feel lucky in my life to have you all as my dear friends and co-workers.

This dissertation is dedicated to my dearest dad and mom. I know it is a quite long distance for family union these two years, but it’s very happy every time I call you and shared new updates with each other. This experience can’t be more meaningful for growing up be a responsible person. I couldn’t accomplish these without having you always standing by myside with loves, understandings, and supports. Love you forever! Life is full of challenges and funs, I believed that nothing is worth afraid of as long as trying your best and believe in yourself!

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CHAPTER 1

Introduction

Food colorants as a main food additive are used for compensating the food color loss after food processing, and food products applied with food colorants can attract consumers as long as the perceived color fulfilled their expectations [1]. As a typical example, red food colorant was added in Maraschino cherry after bleaching with sulfite brine after processing. Anthocyanins from fruit and vegetable as one of the natural pigments have received great attention lately for their clean label (from natural plant source), abundant resources, and supplementary pharmacological effects including cardiovascular disease prevention [2], anti-inflammatory effects [3], etc. The intrinsic high antioxidant property of anthocyanins is attributed to its role as a reactive hydrogen donor or an electron donor in the free radical reaction against the oxidative damages for human body [4]. However, anthocyanin pigments with broad color range (from orange, red, to blue and purple) are unstable under processing and storage conditions, such as heat, high pH and exposing to the light and oxygen [5]. These factors induce the pigment degradation by breaking down the stable anthocyanin structure into colorless phenolic acids and aldehyde [6].

Anthocyanins are identified as flavonoid, in the subclass of polyphenols. The bright red color of anthocyanins in the aqueous phase is mainly related to its pH-sensitive cationic flavylium structure in acidic environment. The classification of an individual anthocyanin is determined by the type of anthocyanidin (aglycone base), the number, type and attaching position of sugar moieties, and the possible acylation of the sugers [7]. This water-soluble pigment is widely available in vascular plants, such as flowers, fruit and vegetables, whereas their distribution varied among different plant materials [8]. Different with the ones from other plant sources, anthocyanins in fruit mostly existed in the form of non-acylated 3-O-glycoside, and are more liable to the environment [9]. Thus, maintaining and stabilizing anthocyanins during food processing and storage are case by case for different fruit varieties.

Extraction is the first processing step for obtaining anthocyanin pigments, and the extraction efficiency could influence the following production and analysis. The typical extraction procedure applies acidic organic aqueous solvent (water with alcoholic solvent) marinating the homogenized plant materials for transferring the polar anthocyanin pigments into the solvent with similar polarity. The extraction efficiency may be facilitated by various extraction methods and apparatus. For example, conventional solvent extraction (CE) utilized heat as energy source, whereas modern extraction methods can apply more advanced energy to break down the plant cell walls, including ultrasound, microwave, high pressure, supercritical fluid, etc.[10] The optimal conditions considering the specific extraction method and fruit variety were investigated individually in some studies [11, 12]. Key extraction parameters include solvent type, solvent concentration, temperature, time and solid to solvent

ratio [5, 13]. The main analytical measurements for determining the optimal extraction conditions are aimed to achieve high total monomeric anthocyanin (TMA), total phenolic content (TPC) and DPPH radical scavenging activity (DPPH), as well as high percentage polymeric color (PPC). Although many studies have evaluated the optimal extraction conditions using these chemical quantification analysis, limited studies reported the correlation between the extraction conditions and the distribution of specific individual anthocyanins in the obtained extracts [8, 11].

Due to the sensitivity of anthocyanins to the environment conditions, storage of the anthocyanin extracts has been a big challenge. To stabilize anthocyanins during storage and to extend their shelf life, microencapsulation technique may be applied as it can immobilize the droplets and entrap anthocyanins extracts inside a colloid vehicle (microcapsule) to fully protect it from external factors and allow the control of its release [5]. Ionic gelation is the simplest method for forming the microcapsules by electrostatic interaction between the adverse charged wall materials [14]. The naturally existed biodegradable poly-cation, chitosan (CH), has been commonly used as the main encapsulating agent for its outstanding gelling and water holding ability [15]. With the addition of inorganic anionic crosslinking agent like alginate, sulfate, citrate and tripolyphosphate (TPP), a hydrogel may be formed to encapsulate the active compounds [16]. Recently, a newly found rod shape nano-material called cellulose nanocrystals (CNC) has attracted increasing attentions. CNCs are prepared from sulfuric acid hydrolysis for native cellulose fibers from wood pulp. The hydrogels formed from CH-CNC may be a good drug delivery vehicle with remarkable mechanical properties [17]. It is worthwhile to investigate this

new anionic crosslinking agent for microencapsulating anthocyanin extracts in comparison with other previously reported crosslinking agent because the use of CNC as a degradable biopolymer may fix the current limitations.

Therefore, the overall goal of this thesis research was to investigate the optimal extraction conditions and microencapsulation technique for extracting and stabilizing anthocyanins and polyphenols from fruit materials. The specific objectives were to evaluate optimal extraction conditions based on one conventional and one advanced extraction method for three typical anthocyanin rich fruit (blueberries, cherries and red pear peels) with different individual anthocyanin compositions, and then to evaluate the microencapsulation formulation and procedures using one inorganic anionic counterion (TPP) and one organic poly-anion (CNC) on chitosan based microencapsulation for stabilizing blueberry anthocyanin extracts. The optimal extraction conditions were first determined by chemical assay, and then further evaluated through HPLC analysis to investigate the influence of the identified extraction conditions on the distribution of specific individual anthocyanins in the different fruit extracts. The microencapsulation formulations and procedures for the two different crosslinking agents were determined individually through a continuous 3-step study on titration direction, concentration of anionic crosslinking agent, and the amount of loaded blueberry anthocyanins. The difference between the encapsulation formulations from two different crosslinking agents were then compared on their encapsulation efficiency and capsule characteristics. This study provides a systematic approach to prepare and storage anthocyanin extracts with stabilized structures and also promotes a new material for anthocyanin encapsulation.

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CHAPTER 2

Literature review

2.1 Introduction

Food colorant is an important additive that can influence the perceived flavor, quality, and freshness of any food products. North America dominates the food colorant market, followed by Europe and Asia regions. With an estimated value of $2.3 billion by 2019 [1]. There are growing concerns for synthetic food colorant and their potential linkage with the hyperactive behavior of kids [2], the natural food pigments from fruit and vegetable sources are preferred by consumers. Amongst these natural food pigments, water-soluble anthocyanins are the second largest natural food colorant followed by lipophilic carotenoid. According to the US Food and Drug Administration (FDA), 4 anthocyanin-derived colorants, including grape skin extract, grape color extract, fruit juice, and vegetable juice, approved for food usage [3]. They are commonly used in the low pH foods or beverages as major red, purple, and pink hue and a few blue shades exist at lower pH [4].

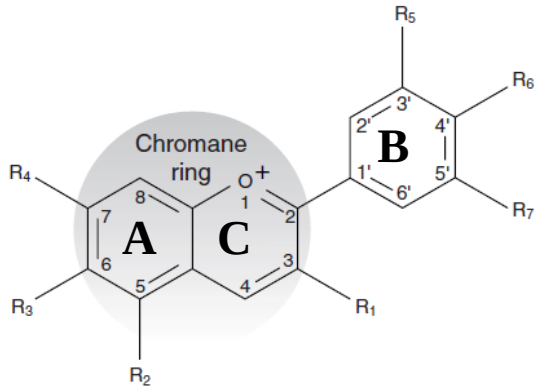
2.2 Overview of anthocyanins

2.2.1 Definition of anthocyanins

The term, anthocyanins, originates from the Greek Anthos, a flower, and kyanos, a dark blue color. They are polyphenolic compounds widely available in vascular plant materials, such as flowers, fruit and vegetables, and classified as flavonoids. The main structure is based on a C15 skeleton consisting of a chromane

ring bearing a second aromatic ring B in position 2; the cyclic structure following the C6–C3–C6 pattern (see the structure in **Table 2.1**) [5]. The whole flavylium cation is named anthocyanidin. There are six common anthocyanidins that exist in nature, with differently distributed hydroxyl and methoxyl groups over ring B (**Table 2.1**).

Table 2.1 Basic structures of six common anthocyanidins (aglycone of anthocyanins), their relative form of 3-O-glucoside anthocyanins and their maximal absorption wavelength (nm) in acidified methanol



Anthocyanidin	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**	**R_7**	**$\lambda_{max\text{-}vis}$ (nm)**
Pelargonidin (Pg)	OH	OH	H	OH	H	OH	H	520
Cyanidin (Cy)	OH	OH	H	OH	OH	OH	H	535
Delphinidin (Dp)	OH	OH	H	OH	OH	OH	OH	546
Peonidin (Pn)	OH	OH	H	OH	OCH_3	OH	H	532
Petunidin (Pt)	OH	OH	H	OH	OH	OH	OCH_3	543
Malvidin (Mv)	OH	OH	H	OH	OCH_3	OH	OCH_3	542
Anthocyanin	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**	**R_7**	**$\lambda_{max\text{-}vis}$ (nm)**
Pelargonidin 3-glucoside	OGlu*	OH	H	OH	H	OH	H	516
Cyanidin 3-glucoside	OGlu	OH	H	OH	OH	OH	H	530
Delphinidin 3-glucoside	OGlu	OH	H	OH	OH	OH	OH	543
Peonidin 3-glucoside	OGlu	OH	H	OH	OCH_3	OH	H	536
Petunidin 3-glucoside	OGlu	OH	H	OH	OH	OH	OCH_3	546
Malvidin 3-glucoside	OGlu	OH	H	OH	OCH_3	OH	OCH_3	546

* Indicates glucose moiety bound with anthocyanidin through glycosidic linkage.
Modified from reference [6, 7]

Due to structural differences, specific anthocyanidins can produce different colors ranging from orange-red (pelargonidin) to blue-violet (delphinidin) at pH 1.0 [7]. The maximal absorption wavelength ($\lambda_{max\text{-}vis}$) is a reading from spectrum which implicates the wavelength with the highest absorbance, this wavelength is commonly

used for detecting water-soluble compounds with specific chemical structures solubilized in aqueous media. Among different anthocyanidins, hydroxylation generally induces a bathochromic shift ($\lambda_{max\text{-}vis}$ moves towards smaller wavelength), while methylation of hydroxyl groups reverses this trend and induces a bluer color [3]. The structural difference also alter the stability of color, as methylation of anthocyanidins induce the better color stability, and this assumption was proved by Harborne since only malvidin derivatives (highly methylated anthocyanidin) were accumulated while all other anthocyanidins lost their amount under high temperature [8].

However, the presence of reactive phenolic hydroxyl groups on some anthocyanidins makes it impossible for the non-methylated anthocyanidins to exist as aglycone forms in nature. Many sugars can bind with anthocyanidins (glucose, galactose, galactose, arabinose, xylose, rutinose, and sambubiose), bindings may be in different numbers of sugar (mono-, di-, and tri-saccharide), and different positions of glycosidic linkage. Glycosidic substitution on anthocyanidin can occur on position 3, 5, or 7 over chromane ring, although 3-O-glucoside is still the most common form. In addition, since anthocyaninsnaturally exist in acidic environments (plant vacuolar sap ranging from pH 3 to pH 7), the sugar moiety (mostly on position 3) may be further acylated by aromatic acids like caffeic, ferulic, and p-coumaric acids, and sometimes acylated by aliphatic acids such as malonic and acetic acids [9]. Commonly, the glycosylation and acylation of anthocyanins induce a more stable structure [10]. The acylated anthocyanins are also found to be more resistant to external factors such as light [11], thermal processing [12], and high pH [13]. For supporting that, a clinical feeding

study of raw and cooked purple carrots reported higher non-acylated anthocyanin recovery in urine and plasma after 24 h of feeding which means higher biocompatibility showed for non-acylated anthocyanins [14]. Furthermore, tri-glycosylated and di-glycosylated anthocyanins are prove to be more stable than mono-glycosylated anthocyanins. For other structural effects, Stintzing et al. investigated the effects of sugar substitute of anthocyanins on the anthocyanin color of blackberry, elderberry, black carrot, red cabbage, and sweet potato, and found negligible color influence on hue for the anthocyanins attached with different types and numbers of sugars at C-3, but additional glycosylation on C-5 resulted in a slight shift to more red-purple color [15].

2.2.2 Potential health benefits of anthocyanins

Considering the wide distribution of anthocyanins in flowers, fruit and vegetables, the daily intake of anthocyanins in the United States was estimated to around 200 mg, about 9-fold higher than that of other dietary flavonoids[16]. Remarkable pharmacological effects have been reported with the consumption of anthocyanin-rich foods, such as reducing the risk of some chronic diseases like cardiovascular diseases [17], cancers [16], and type 2 diabete [18]. In fact, these health benefits are highly correlated to the high antioxidant capacity from anthocyanin compounds. Just like other polyphenols, the anthocyanins act as an active radical scavenging agent and reducing agent with inhibition to the oxidative enzymes [19]. Their antioxidant property can be modulated as the increase of hydroxylation on anthocyanidins could promote an antioxidant response, while the single aglycone with much weaker antioxidant activity [20].

2.2.3 Sources of anthocyanins and their distributions

Anthocyanins are widely available in mature plants, mostly present in peels, leaves, stems and other storage organs of plants. **Table 2.2** lists the concentration and distribution of major and minor individual anthocyanins in some common fruit and vegetables. It is obvious that the majority red, orange fruit are rich in glycosides of cyanidin and pelargonidin, while the blue, purple colored food are more abundant in delphinidin based anthocyanins. Overall, fruit, especially the berry fruit are the most anthocyanin-rich plant sources [21–23], whereas for other fruits, anthocyanins are not distributed in the entire portions of the fruit. Although the small soft fruit like blueberries and cherries possess anthocyanins both in flesh and peel [24], anthocyanins in pome fruit such as apples and pears, are most likely located in the their peels (close to the epidermal layer) [25,26]. Other factors including the cultivars of the same fruit [27], the maturity of fruit [28], growing season and location [29] also contribute to the concentrations of anthocyanins in the fruit materials.

Table 2.2 Anthocyanin (ACN) concentration and individual anthocyanin distribution in some common fruit and vegetables

	Major anthocyanins	Minor anthocyanins	ACN*
Fruit			
Apple	Cy-3-glucoside	Pn-3-glucoside	1.3-12.3
Strawberry	Pg-3-glucoside	Cy- 3-glucoside, Pg- 3-rutinoside	21.1- 41.7
Blackberry	Cy-3-glucoside	Cy-3-rutinoside, Mv-3-glucoside	245.68- 300.5
Blueberry	Dp-3-glucoside	Cy, Pn, Pt and Mv mono-glucoside	386.5- 486.5
Raspberry	Cy-3-glucoside	Pg-3-glucosides, Pg-3-rutinoside	92.1
Sweet cherry	Cy-3-rutinoside	Cy-3-glucoside, Pn-3-rutinoside and Pg glucosides	122
Black currant	Cy-3-rutinoside	Cy 3-glucoside, Dp, Pt, Pg and Pn glucosides	476
Plum	Cy-3-glucoside	-	19
Red pear peel[25]	Cy-3-galactoside	Cy-3-glucoside	0.56-2.43
Grape	Dp-3-glucoside	Cy, Pn, Dp, Pt and Mv mono- and di-glucosides, free and acylated	26.7-120.1
Vegetables			
Red onions	Cy 3-glucoside	Dp 3-glucoside, Pt-3-glucoside	48.5
Red cabbage	Cy 3-glucoside	-	322
Eggplant	Dp-3-glucoside	-	85.7
Black bean	Dp-3-glucoside	Pt and Mv monoglucosides	44.5

* ACN unit is milligrams of anthocyanin on weight basis.
The corresponding anthocyanidins are Cy = Cyanidin, Dp = delphinidin, Mv = malvidin, Pg = pelargonidin, Pn = peonidin, and Pt = petunidin.
Modified from references [10, 25, 30].

2.3 Extraction of anthocyanins from plant materials

Exaction is an important step for obtaining anthocyanins from plant materials. It can influence the production of colorant and also the color quality. Theoretically, three key components are needed in the extraction process: raw plant material; solvent for maceration (similar polarity with anthocyanin); and energy (facilitating the mass transfer of bioactive compounds from plants into the solvent).

To make sure the high reproducibility for anthocyanin extraction, defatting process are also needed for the oily plant materials. The raw materials are suggested to be homogenized by liquid nitrogen [6]. The smaller the particle size, the more release of anthocyanins from the raw materials. Anthocyanins are water-soluble and pH

sensitive. Hence, solvents with similar polarity are preferred to help with mass transfer. Methanol, ethanol, acetone and water are the most common solvents used to extract anthocyanins [31]. To modify the polarity, more than single solvents may be mixed together as a co-solvent for increasing the solubility of anthocyanins with various structures (**Table 2.3**).

Table 2.3 The factors influencing the polarity of solvent and anthocyanin by different structural characteristics and examples in anthocyanins extraction

Factors	**Characteristics**	**Specific examples for polarity**
Solvents	Related to the dielectric constants δ as polar (δ > 50), semi-polar (δ = 20 - 50), or non-polar (δ = 1 - 20); the higher the temperature, the lower the dielectric constant.	Water > Methanol > Ethanol > Acetone
Anthocyanidins	-OH groups in the B-ring ring increase the polarity, -OCH_3 decrease the polarity	Delphinidin > Cyanidin > Petunidin > Pelargonidin > Peonidin > Malvidin.
Glycosylation of anthocyanidins	Increased in the polarity: Anthocyanin > Anthocyanidin; the identical sugar substitute attribute to the same polarity; 3, 5 glycosides > 3,3 glycoside >3 glycoside; 3, 5 glycosides > 3.7 glycoside.	Monosaccharides: Galactoside > Glucoside > Arabinoside > Xyloside>Rhamnoside. Disaccharides: Sophoroside > Sambubioside > Rutinoside
Acylation of sugar on anthocyanins	Decrease in the polarity.	Malic acid> Acetic acid > Malonic acid > Succinic acid

Summarized from references [32, 33].

Although methanol and acetone present good extraction efficiency in many studies [34], their toxicity remains a major concern, which limits their usage. The addition of acid in solvents is an alternative way for avoiding the usage at large amount of organic solvents, as 0.001% HCl addition into ethanol could reach 80% extraction efficiency in comparison to using only methanol as solvent [6]. However, considering the possible structural breakdown from strong acid hydrolysis, weak

acids are recommended for the use of anthocyanin extraction [5]. Also, the low pH of solvents helps to stabilize anthocyanins in the flavylium cation (AH^+) (**see session 2.2.1.1**).

In addition of the anthocyanin available in raw materials, the particle size of plant materials and the solvent type, different extraction techniques employed could also greatly influence extraction efficiency. Depending on the energy sources and apparatus, there are two major types of extraction methods: conventional solvent extraction and advanced solvent extraction. The conventional solvent extraction is based on the choice of solvent coupled with the use of heat and/or agitation [35]. The advanced extraction method introduce the new energy like sonication, microwave, supercritical fluid, and high hydrostatic pressure with the purpose of improving the extraction efficiency by increasing the mass transfer. The later could reduce the organic solvent portion and extraction time.

2.3.1 Conventional solvent extraction (CE)

By using conventional solvent extraction, solids are mixed with the solvent at 50-90 ℃ for several hours or several days at ambient temperature. Methanol is still the most common and effective solvent for extracting anthocyanins using this method [36, 37], even though the growing concern on their toxicity and possible contamination in the extracts. Ethanol is a good substitute with high extraction capacity for soluble bioactive compounds. In addition, the higher temperature and lower solvent to solid ratio for ethanol were found to promote polyphenolic extraction from grape pomace [38]. The concentration of organic solvent in extraction solvent also matters. A study on grape marc phenolic extraction optimization found the decrease on remaining

phenolics in extracts when water content was above 50% in the solvent [39]. Generally, 60-80% concentration of organic solvent is used in this extraction method. However, at least 1 hour is required for this extraction method. Hence, other advanced methods have been developed to reduce the extracting time.

2.3.2 Ultrasound-assisted extraction (UAE)

Compared to the conventional method, UAE is one of the most simple and inexpensive extraction method. It is more easy to handle since sonication bath is available anywhere and operation is easy and fast. Ultrasonic radiation uses frequencies higher than 20 kHz and can produce sound waves and create cavitation bubbles near the sample tissue. The expansion and compression cycles formed during the wave travelling in the solvent media can produce negative pressure and high-speed jets of liquid, thus having strong impact on the solid surface for disrupting cell walls and releasing more cell contents [40]. Similar solvent concentration and solvent type as CE are used in this method. With the assistance of sonication, shorter time (20-60 min) and lower heating temperature can be used to achieve the same or better extraction compared with CE. The optimal conditions for phenolic extraction from orange peels were identified at 40 ℃, sonication power of 150 W and 80% ethanol [41]. Former studies reported higher anthocyanins content achieved together with high retention of total phenolics and flavonoids in purple sweet potato extracts using ultrasounds extraction [36]. Therefore, UAE may be an ideal extraction method for anthocyanins by using mild processing conditions and helping to reduce the loss of less stable non-acyl and mono-acyl anthocyanins.

2.3.3 Microwave-assisted extraction (MAE)

Microwaves is a non-ionizing radiation with frequencies between 300 MHz and 300 GHz. The radiation to the plant materials can cause the molecular motion in matrix or solvents with dipoles (water molecule), thus resulting in the whole sample heating [42]. The heating accelerates the evaporation of moisture in cells and promote the generated steam to swell and eventually rupture the cells, thus releasing their active components. From a study for optimizing polyphenols extraction from red grape pomace, MAE was proved more efficient than CE and UAE, since microwave increased the mass transfer through diffusion inside the solid and decreased the extraction time [43]. By using MAE, using water only as the solvent could reach the same extraction efficiency as using 50% ethanol. However, due to the application of high power (100- 600W), MAE can cause a high temperature around 80-150 ℃ [6, 44], in which the heat-induced hydrolysis effect may occur. A study on Chinese bayberry anthocyanin extraction mentioned that cyanidin-3-O-glucoside degraded into cyanidin without influencing the antioxidant activity of the extracts under MAE [45]. This result could be related to the dipoles of polyphenols (due to their hydroxyl groups) helping absorb radiation.

2.3.4 Supercritical fluid extraction (SFE)

Supercritical fluid (SCF) refers to a type of solvent that forms when the temperature and pressure of the fluid increase above its critical point. For example, water can transfer into SCF phase at 221.19 bar and 374.3 ℃. The common SCFs includes methane, carbon dioxide, ethanol, benzene, and water [6]. SCF has the advantage of both high penetration power from the gas state and high density from

the liquid state to facilitate the extraction process [46]. CO_2 is a perfect SCF used in SFE (induced at 73.87 bar and 31.2 ℃) due on its non-reactive and inflammable property compared to other solvents. It is also cheap and easily recycled. SFE extracts are also generally recognized as safe (GRAS) to be used in food products, making SFE a very promising technique for food processing [47]. SFE is very suitable for extracting heat-sensitive compounds like anthocyanins. The low pH with the generation of carbonic acid has a positive impact on the stability of anthocyanins as well [48]. However, the non-polarity of CO_2 makes it inappropriate to use for the mass transfer of polar phenolic compounds. Extra addition of polar co-solvents such as ethanol, methanol, ethyl acetate and acetone are recommended. In the optimization study of grape peel anthocyanin extraction, the optimal SFE conditions were identified as 45–46 ℃, 157-162 bar of pressure with 6–7% ethanol as a modifier [47]. In the use of SFE, temperature and pressure both had significant effects on extraction efficiency while not much influence due to the addition of 5-8% of ethanol [49]. In terms of anthocyanins, extraction pressure was a more important variable affecting the recovery, as it decided the density of the extraction fluid at extraction temperature below 50 ℃ [50]. The only concern for using this technique is the high cost of the machine.

2.3.5 High hydrostatic pressure (HHP) assisted extraction

HHP is another non-thermal technique utilizing super-high hydraulic pressure (1,000–8,000 bar) that leads to an intense mass transfer at ambient temperature (~20 ℃) and shorter time (<15 min)[51]. The application of HHP can increase cell permeability by creating a huge pressure difference between two sides of the cell

membranes [6]. Shouqin et al. applied HHP on propolis extraction and obtained significantly high flavonoid content compared to CE [52]. Another study on grape by-product extraction also showed that the acylated anthocyanins were more favorably extracted by HHP [43]. The extra high pressure is the key factor in this technique, for example, anthocyanin extracts from raspberry [53] and strawberry [54] both showed that the higher pressure resulted in the least loss of anthocyanins. A study on HHP extracted tomato and carrot purée also suggested the less degradation of phenolic acids and higher lightness in color was noticed in comparison to the thermal processing [55]. Anthocyanins from grape skins using HHP at 6,000 bar and 70 ℃ induced the highest extraction efficiency compared with hot water, ultrasonic, and pulse electric field extractions [56]. Similar to SFE, the concern for using this technique is the high cost of the apparatus.

2.3.6 Other extraction methods

Pulsed electric field (PEF) is another non-thermal method with the application of microsecond pulses of high electric field strength. In the operation, the cell membranes of the plant materials placed between the two electrodes, are broken down, thus increasing the release of phenolic compounds. PEF can be operated continuously at room temperature in only a few seconds to avoid degradation during processing. However, PEF is usually used as a sample pretreatment. López et al. applied PEF with 5 kV/cm, 2.1 kJ/kg to the grape pomace before the maceration for wine making, and found that PEF could enhance mass transfer by electroporation of plant cell membranes and soften the tissues to increase color intensity of wine [57].

Another study also found that anthocyanin mono-glucosides can be more easily extracted by PEF [56].

Accelerated solvent extraction (ASE) is an expensive automated technique using similar organic solvents used in CE to extract bioactive compounds. ASE usually runs under nitrogen at high temperature or under high pressure with the advantage of rapid mass transfer and prevention of oxygen contact during the extraction. Using 68 bars of pressure, one extraction cycle, and high temperatures of 80, 100, or 120 ℃, the higher total anthocyanin content was extracted in red grape pomace than that of using 50 and 70% ethanol as solvent in CE [58].

Enzymatic treatment for plant materials before extraction is an alternative pretreatment for breaking the hydrophilic and hydrophobic binding between phenolic compounds and plant cell walls [59], thus helping the release of phenolics. Previous studies reported an increase of phenolic compounds in the extracts with the addition of β-glucosidase on cranberry pomace [60] and commercial pectinase and protease on black currant pomace [37]. However, although more phenolic compounds were achieved by enzymatic extraction, none anthocyanin increases were founded [61]. In addition, in comparing UAE technique with enzymatic extraction of phenolic compounds from acerola fruit, much higher yield of phenolics with higher antioxidant capacity in the extracts were still achieved by using UAE method [62].

2.4 Stability of anthocyanins

2.4.1 The influencing factors for anthocyanin stabilization

2.4.1.1 Type of solvent, concentration and chemical structure of anthocyanins

Anthocyanin extracts are highly unstable and very susceptible to degradation under various circumstances, such as pH, temperature, light, oxygen, type of solvents used for extraction, chemical structures, the presence of enzymes, and other accompanying substances (flavonoids, proteins and metallic ions) [63].

Based on the solvents summarized in **session 2.3** and their relation with anthocyanin structures described in **session 2.2.1**, the red anthocyanin pigments are favored by the amount of anthocyanin flavylium (AH^+) in the aqueous system and the solubility in the specific solvent. For example, the red anthocyanin pigments can be observed in more polar solvents while yellow anthocyanin pigments in aprotic solvents. This phenomenon is related to the monomer and polymer form of AH+ in the solvent, respectively [5].

2.4.1.2 pH

Anthocyanins are water soluble and highly pH dependent. In a aqueous system, the binding strength varies with pH (particularly pH 4-6) due to transformation in the relative concentrations of different anthocyanin species, namely flavylium cation (AH^+), hemiacetal (B), chalcone (C), quinoidal bases (A) and anionic quinoidal bases (A^-) (**Fig. 2.1**). As mentioned before, the red colored anthocyanins are due to the existence of AH^+ which is mostly dominant at pH<3, while at pH 4-6, all anthocyanins co-exist, but the A is the major unstable species at pH 4–6, the A- can formed when pH reaches 7, when the ionic interactions take an critical role in the crosslinking of anthocyanins to other cations [64]. Another anthocyanin species transformation happened between AH^+ and A when pH changes from 1.0 to 4.5 together with hydration. This is a reversible process for monomeric

anthocyanins, and has nothing to do with the polymerized anthocyanin pigments and non-enzymatic browning pigments which are excluded from the absorbance calculation. This is also the main principle for measuring total monomeric anthocyanin (pH differential method) [65]. The degradation of anthocyanins is induced by the production of B and C. The de-coloration could happen just after 41 h in room temperature. As C accumulates through continuous open-ring process from other species (accelerated degradation when pH>7), the whole transformation equilibrium goes to an end and reverse reactions stop [66].

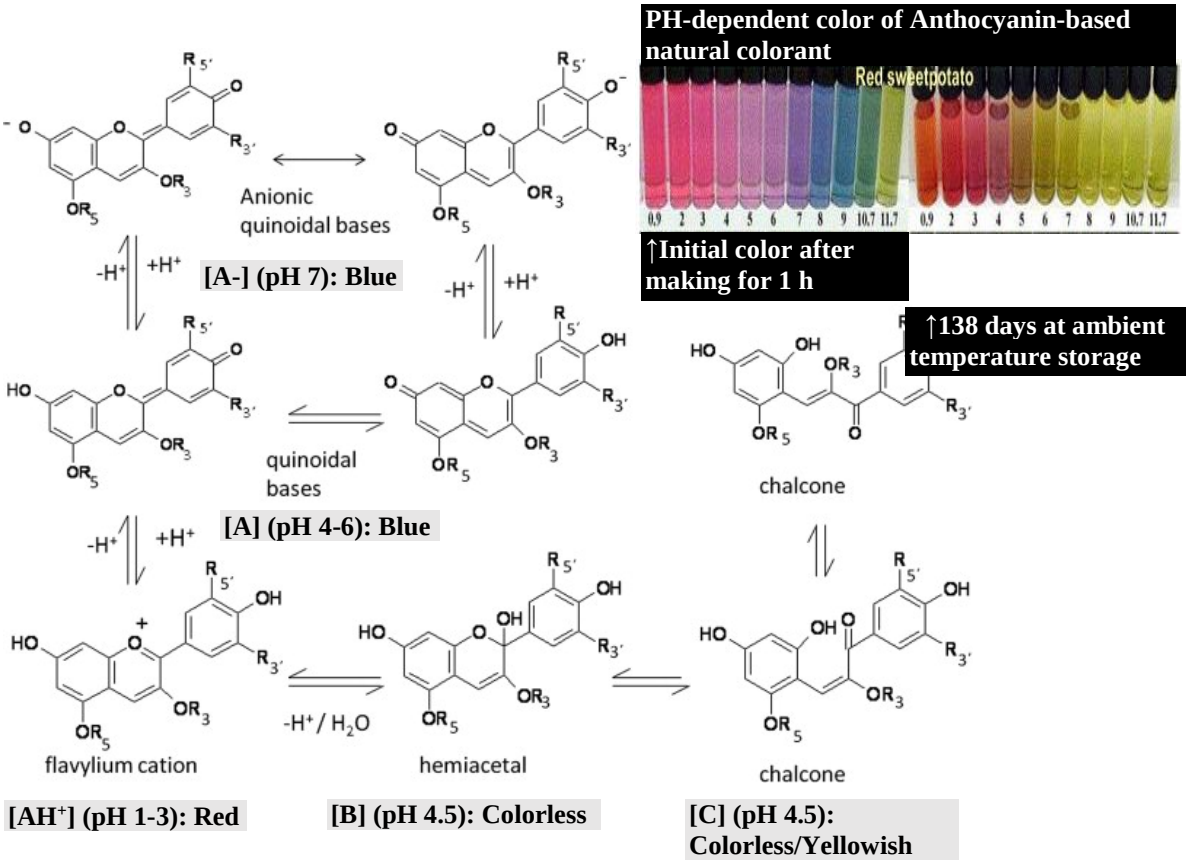


Fig. 2.1 Anthocyanin species transformation in aqueous system with pH dependent equilibrium (Modified from references[3, 67, 68]).

2.4.1.3 Storage temperature

The stability of anthocyanins greatly decreases during processing and storage as temperature rises [10]. The degradation is primarily caused by the cleavage of covalent bonds and the oxidation of anthocyanins accelerated by thermal process. The formation of various intermediate compounds are correlated with the structural nature of anthocyanins and heating method.

Two proposed mechanisms for thermal degradation on Pelargonidin-3-glucoside and Cyanidin-3-glucoside. The first one operates at pH 1.0 with 7 hour of heating at 95 ^{o}C and the intermediate compounds were monitored by HPLC-DAD-MS data [12]. A degradation scheme started from de-glycosylation of anthocyanins followed by the cleavage of flavylium structure with generation of small phenolic acids or aldehydes. However, another study on the degradation pathways at pH 3.5 found that anthocyanins were decomposed upon heating into a chalcone structure firstly, then leading to a further transformation into a coumarin glucoside derivative with the loss of the B-ring [69]. Also, for acylated anthocyanins, acyl-glycoside moieties were able to split off directly from the flavylium backbone. (**Fig. 2.2**)

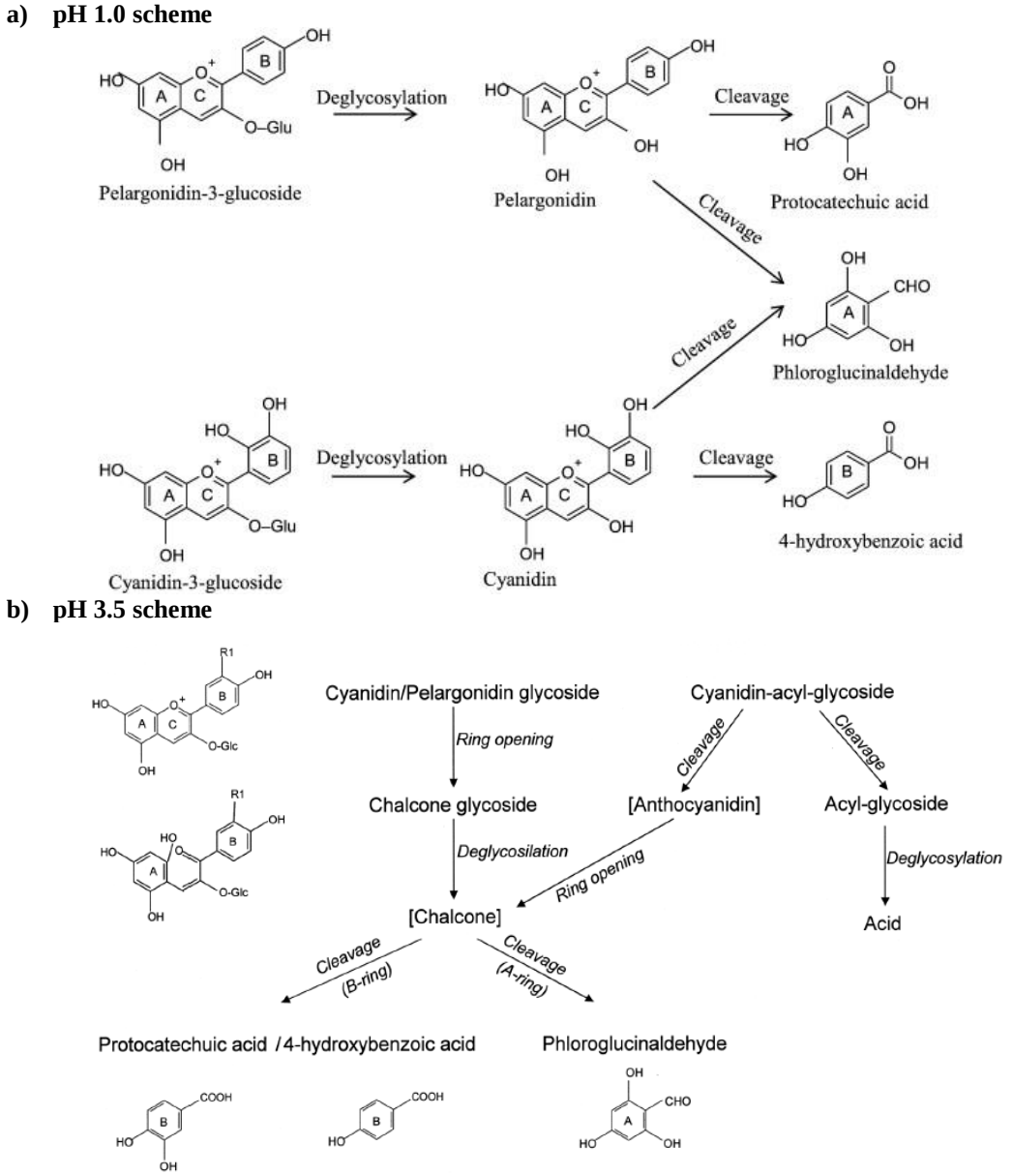


Fig. 2.2 Proposed mechanism for thermal degradation of two common anthocyanins (Modified from references[10, 68, 69]).

2.4.1.4 Oxygen

Oxygen plays a critical role in anthocyanin degradation. Without oxygen, even under the thermal processing, anthocyanin degradation does not happen [63]. The presence of oxygen leads to the degradation through a direct oxidative mechanism (**Fig. 2.3**) and/or through the action of oxidizing enzymes (such as PPO) [10]. This

effect is strengthened when the oxidant agent (H_2O_2 or O_2) is accompanied by ascorbic acid [6].

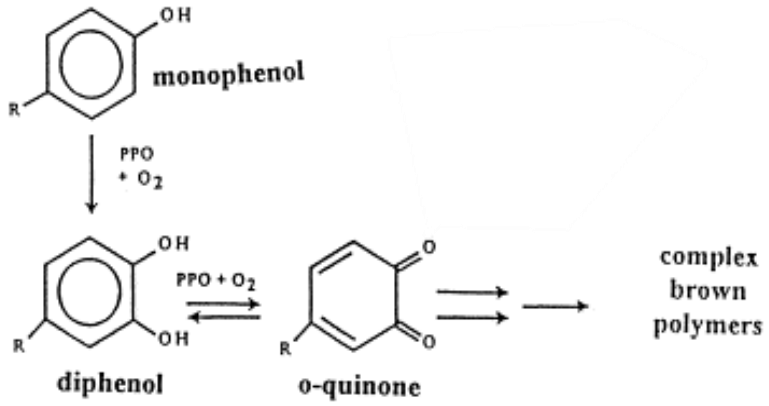


Fig. 2.3 Enzymatic oxidation mechanism induced by polyphenol oxidase (PPO) (Modified from reference [70]).

2.4.1.5 Enzymes

Enzymes, such as polyphenol oxidase (PPO) also plays an important role in the browning of the anthocyanin pigments. With the presence of substrates, in the phenolic solution (caffeic acid, chlorogenic acid, or gallic acid), the o-diphenolic compounds can be involved in the first step of polyphenolic oxidation [71]. The acid could further oxidizes into its o-quinone, which can react with anthocyanins to form unwanted browning polymers [10]. In this case, since the quinone formation from acid is vital in polyphenol oxidation process, the loss of anthocyanin pigments can be prevented by reducing the amount of quinone [71]. Another enzyme, glycosidase, can also directly break glycosylic bond between sugar residues and the aglycone of anthocyanins, thus forming unstable structures [63].

2.4.1.6 Light

Anthocyanins are generally unstable under UV light, especially in the presence of sugar [72]. In addition, C-5 glycosylated anthocyanins seemed more susceptible to photochemical decomposition than other forms of anthocyanins [6].

2.4.1.7 Ascorbic acid

Ascorbic acid (AA) is a negative stimulus on anthocyanin stability, and its presence accelerates the degradation of anthocyanin pigments with oxygen[73, 74]. Under aerobic conditions, the existence of transition metals (e.g. copper ions) can fasten the degradation of AA and anthocyanins when they are both present [75]. The degradation mechanism may be related to the condensation of AA on the C-4 of the anthocyanin structure, causing the mutant degradation of them through the oxidation [73]. Another explanation was proposed on hydrogen donor role of AA and their degradation product (hydrogen peroxide), the continuous oxidative cleavage of the flavylium ring may happen through a free radical reaction according to Iacobucci and Sweeny [76]. In addition, the deterioration of grape anthocyanins was observed, showing an increase in lightness value and decrease in redness value [74].

2.4.1.8 Sugars

In respect to sugars effect on anthocyanins, former studies reported that the low concentration of sugar (~20%) had a protective effect on anthocyanin content and color density for frozen strawberries [77]. The sugar-induced osmotic pressure can highly reduce the moisture content, thus stabilizing the color. However, in the anthocyanin aqueous extracts, the addition of sugars are known to have a negative effect on anthocyanin pigments. A study by Daravingas and Cain[78] tested four types of sugars (sucrose, fructose, glucose, and xylose), all of them caused anthocyanin

degradation, and their degraded products were able to cause more extreme anthocyanin degradation. These effects were all related to the formation of polymeric compounds or complexes with increasing sugar content in the aqueous system of anthocyanins, thus reducing the effective anthocyanin structures.

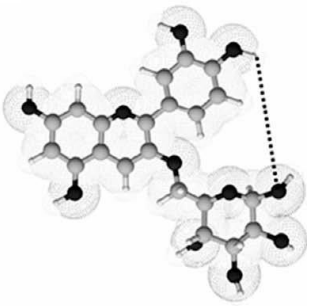
2.4.1.9 Sulphates and sulphites

Sulphates and sulphites used in fruit storage and wine production can produce discoloration of anthocyanins since their addition can promote the formation of colorless sulfur derivatives (attaching at C-2 or C-4 of anthocyanin) [63]. The reaction is reversible, and the flavylium color can be regenerated by acidification and heating of the product.

2.4.2 Anthocyanin association

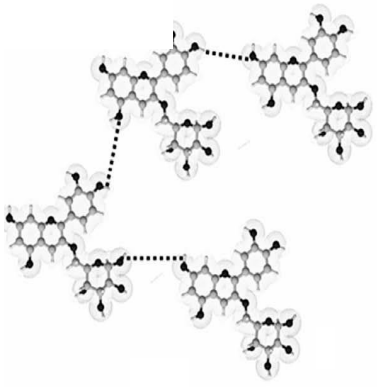
The association of anthocyanins with itself or other compounds affects their pigment stability. Three types of association reactions exist (**Fig. 2.4**): (1) Self-association among anthocyanins; (2) Copigmentation, including intramolecular copigmentation and intermolecular copigmentation; and (3) Metal complexation for their chelating effect on metals with –OH groups of anthocyanins.

A) Intramolecular Copigmentation



B) Intermolecular Copigmentation

C) Self-association



D) Metal Complexation

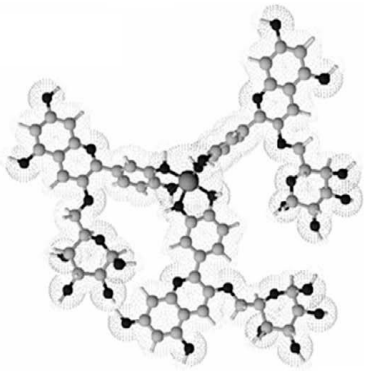


Fig. 2.4 Anthocyanin association (Modified from reference [5]).

2.4.2.1 Copigmentation

The phenomenon of copigmentation was first observed in 1916 by Willstätter and Zollinger, who noticed that the color of a grape pigment (rich in malvidin 3-

glucoside) changed its hue from red to bluer red with the addition of tannin or gallic acid. It was found later that this was due to the molecular associations between pigments and other non-colored organic molecules (co-pigments) in the solution by occupying the reactive phenolic hydroxyl groups over Ring B. Co-pigmentation is commonly used for enhancing the color stability of anthocyanins in wine [79]. The main strategy was to use copigments to form associations to prevent the flavylium cation of anthocyanins from the surrounding nucleophilic attack from water molecule [6].

Both anthocyanin copigmentations can reflect two typical changes on UV-Vis spectrum, the first one is the shift of the maximum absorption wavelength (λ_{max}), identified as a bathochromic effect ($\Delta\lambda_{max}$), resulting in a bluer color with the completion of copigmentation. Another copigmentation is the hyperchromic effect (ΔA), in which anthocyanin intensity is fortified and presented as a more red color [79].

Intramolecular and intermolecular interactions in the copigmentation are inspired from the natural color of flowers. Intramolecular interaction is a stronger covalent linkage between an organic acid, an aromatic acyl group, or a flavonoid with an anthocyanin aromatic structure (**Fig. 2.4 a**). The copigments are normally aromatic groups of hydroxycinnamic acids. The association played as a major role in stabilizing acylated anthocyanins [80]. The efficiency could be different depending on the copigments applied. A study compared three naturally existed polyphenolic compounds on color stability of anthocyanins in açai fruit[81], and found that the application of flavone-C-glycosides induced significant higher hyperchromic shifts at all pH and temperature, compared to the usage of phenolic acids or procyanidins.

Copigmentation can also be achieved by weaker intermolecular interactions, in which colorless flavonoids or other phenolic compounds can bind with anthocyanins through weak hydrophobic forces (**Fig. 2.4 b**). In this interaction, both flavylium cation (AH^+) and the quinonoidal base (A) forms of the anthocyanins are able to participate the conjunction with copigments. Relatively, the interactions are more favored by the non-acylated anthocyanins (abundant in berry fruit). From the study of Eiro and Heinonen [82], among five anthocyanins and phenolic acids, the intermolecular association of rosmarinic acid and malvidin 3-glucoside resulted in the highest degree of bathochromic shift and hyperchromic effect. Another study even reported delayed anthocyanin degradation together with the less loss of ascorbic acid by copigmentation with rosemary polyphenolics [74]. The same study pointed out that the copigmentation could prevent anthocyanin polymerization with ascorbic acid.

2.4.2.2 Self-association

Stability of anthocyanins can be achieved by self-association as long as more than one anthocyanins are bonded (**Fig. 2.4 c**). This could attribute to the effect of anthocyanin concentration as the increase on total anthocyanins induced a greater color intensity [11]. The mechanism of self-association was described as stacking, in which vertical stacking of the molecules of anthocyanins is formed and stabilized mainly through the hydrophobic interactions between anthocyanins [6].

2.4.2.3 Metal complexation

Metal complexation is rarely used in the food industry with the consideration of possible contamination of food products by metal ions. This association contributes to the blue hues of anthocyanins through a pH-dependent color change (pH 4-6) and

the formation of chelated complexes with bi- and tri-valent metal ions [83]. The metallic linking at hydroxyl groups in the anthocyanin structure is described in **Fig. 2.4 d**. The common metals that form chelated anthocyanin complexes are tin (Sn), copper (Cu), iron (Fe), aluminum (Al), magnesium (Mg), and potassium (K). Ngo & Zhao applied an aqueous system containing stannous (Sn) ion, hydrochloric acid, formaldehyde, and tannic acid to pretreat purple pears, and demonstrated an increasing red color retention on pear peels after thermal process [25].

2.4.3 Encapsulation for anthocyanin stabilization

Except for understanding of internal nature of anthocyanins (chemical structure and association), and the external factors (pH, light, oxygen and heat) that could deteriorate the anthocyanin color quality by the structural transformation, it is important to develop a anthocyanin stabilization techniques to remain their original chemical structure and concentration. Encapsulation technique as a coating method shields the anthocyanins from external factors and can be a perfect choice for stabilizing anthocyanin in the polymer vehicle with mild processing, easy handling property, and precise releasing control.

2.4.3.1 Definition of encapsulation

Encapsulation is a technique in which an active compound is mixed with a carrier agent (or wall material). The active compound (or core material) can be a flavor, drug, or pigment substance. The first application of this technique was invented by Barrett K. Green working on microencapsulating ink and a prototype carbonless paper in 1942 [84]. It has been commonly used in drug delivery system since the capsules can provide a substantial protection through the digestion environment in

human body and target the localized area for enhanced absorption [85]. In food applications, this technique can improve food quality by entrapping functional food substances (colorants, volatile oils and flavoring compounds) with fortified processing tolerance, thus improving the appearance, nutrition and healthy functions of food products [86].

Based on the size of active-loaded capsules, encapsulation is classified as nano- (from 1 to 1000 nm) [87], micro- (1000 nm to 1,000 μm), and macro (>1,000 μm) [88]. In this thesis, to achieve the high dispersive property of the capsules, microencapsulation is discussed. Depending on the morphology of the microcapsules (one core or multiple cores), the microcapsules could be divided by core-shell type, matrix type, and coated matrix type (**Fig. 2.5**)[89].

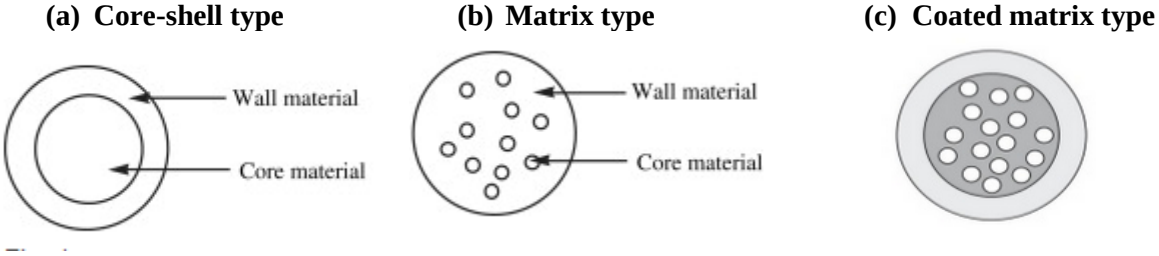
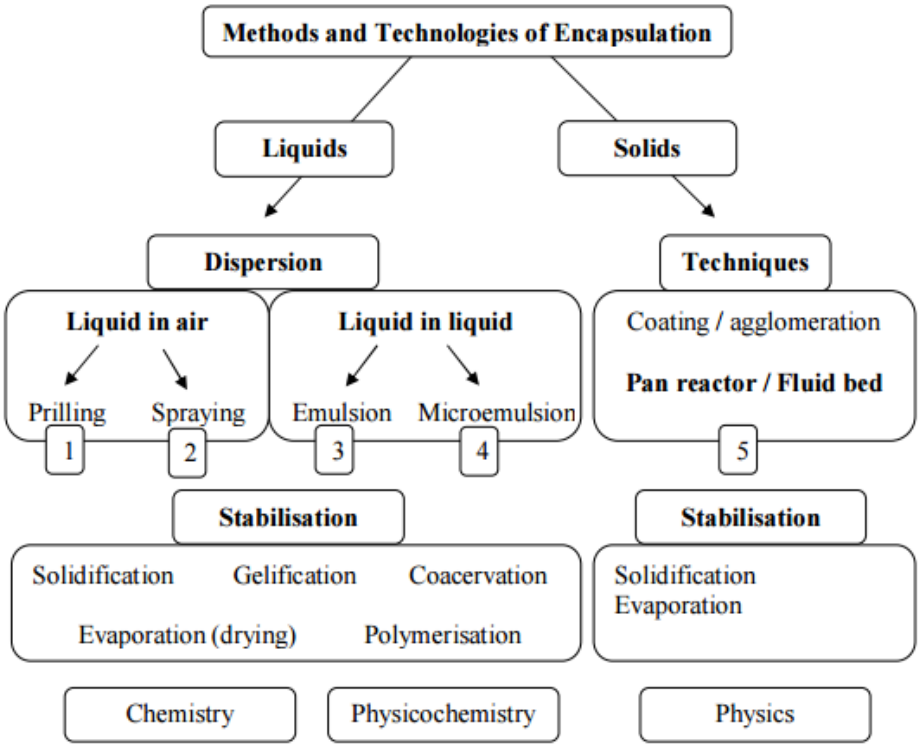


Fig. 2.5 Schematic Diagrams of Main Types of Microcapsules (Modified from reference [89]).

2.4.3.2 Encapsulation methods

There are several techniques that are currently available for encapsulating the active compounds depending on the form of core materials and different capsule forming mechanisms. It can be classified as 1) physical method: spray-drying, fluid bed coating, extrusion; 2) physicochemical method: spray-cooling, hot melt coating, ionic gelation, solvent evaporation and coacervation; and 3) chemical method: interfacial poly-condensation, in situ polymerization, interfacial polymerization, and interfacial cross-linking [89]. (**Fig. 2.6**)



Physical method	Chemical merthod	Physicochemical method
Spray-drying Fluid bed coating Extrusion	poly-condensation in situ polymerization interfacial polymerization, interfacial cross-linking	Spray-cooling Hot melt coating Ionic gelation Solvent evaporation Coacervation

Fig. 2.6 The methods and techniques for forming encapsulation (Modified from reference [89]).

2.4.3.3 Encapsulating materials and different crosslinking principles

Whichever encapsulation method is selected, the main principle for encapsulation is still on the basis that introduces active compounds into a matrix (polymeric wall system) to protect them from environmental conditions [23]. Therefore, understanding the structural property of biodegradable material used for forming the polymeric wall material (encapsulating agent) and the crosslinking mechanism between more than one encapsulating materials is very important for formulating the optimal conditions for encapsulation.

Based on the review from Singh et al. [90], selection of an appropriate wall material can highly influence the physical and chemical properties of the produced microcapsules. The selection of a satisfactory polymer material should consider their stability, volatility, release characteristics, and responses to environmental conditions.

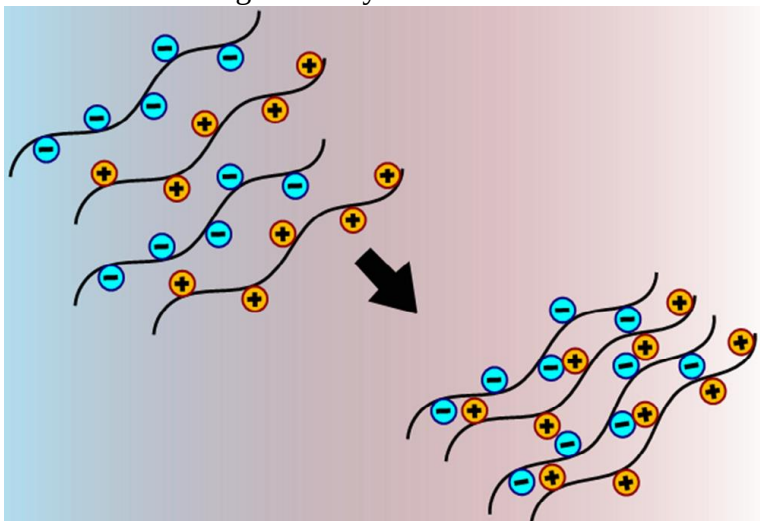
Based on a review of spray-drying encapsulation for food ingredients [91], the typical wall materials for food usage can be divided into three biopolymer groups: natural gums (gum arabic, alginates, carragenans, etc.), proteins (milk or whey proteins, gelatin, etc.), and carbohydrates (starches, maltodextrins and corn syrup solids) and their blends.

Hydrogels are crosslinked polymer network structures capable of swelling and absorbing large quantities of water without dissolution. The formation of polymeric hydrogels between the blends or combination of wall materials has been described by Nguyen et al.[92] The crosslinking could form through either chemical crosslinking (photo-polymerization, click chemistry, enzyme-catalyzed reactions, Schiff's base reactions, and thiol-based Michael reactions) or physical crosslinking induced by

temperature, pH, ionic interaction, guest-host inclusion, stereo-complexation, or complementary binding.

Amongst, the ionic gelation is highlighted as an attractive encapsulation method since it is the simplest way to form a hydrogels without the use of extreme pH or addition of organic solvent [89]. The hydrogel capsules can be achieved by mixing solutions of oppositely charged crosslinking agents to yield a reversible shear-thinning hydrogel system (**Fig. 2.7**).

(a) Schematic mechanism of gelation by ionic interaction



(b) The ionic crosslinking agents used for encapsulation

Cation	**Anion**	**Application**
Calcium	Alginate	Insulin [93]
Gelatin	Sodium alginate	Ginger volatile oil [94]
Chitosan	Alginate	Ampicillin [95], bovine serum albumin (BSA) [96]
	TPP	Tacrine [97]
	Cellulose nanocrystal	Drug carrier [98]

Fig. 2.7 The mechanism and ionic crosslinking agents for encapsulation (Modified from references [92, 99]).

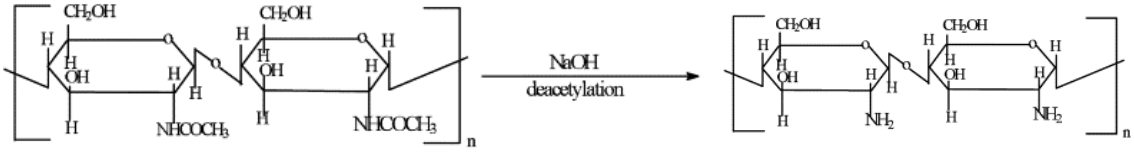
The traditional ionic gelation used the formulation with alginate and calcium to form capsules based on the physically electrostatic attraction between α-l-guluronic acid moieties in alginate and calcium ions, but the gels are usually with less

homogeneousness [100, 101]. A study compared three encapsulation systems on loading bilberry anthocyanins, including PA (system 1, based on pectin amide- CaCl2 crosslinking), WPI (system 2, based on whey proteins), and SL (system 3, based on pectin amide with an additional shellac coating), and the high encapsulation efficiency (up to 99%) was achieved by amidated pectin-$CaCl_2$ forming a shell-core structure owning to the rapid diffusion of the calcium cations, but the particle sizes were much larger than the other two systems [102]. Except the metal ions, the naturally existed biodegradable cationic-chitosan became a promising candidate for anthocyanins encapsulation [103]. And the addition of chitosan can conquer the drawback from the swelling of $CaCl_2$-alginate capsules and retard the release of core compounds from the capsules [101].

2.4.4 Ionic gelation using chitosan as cationic crosslinking agent

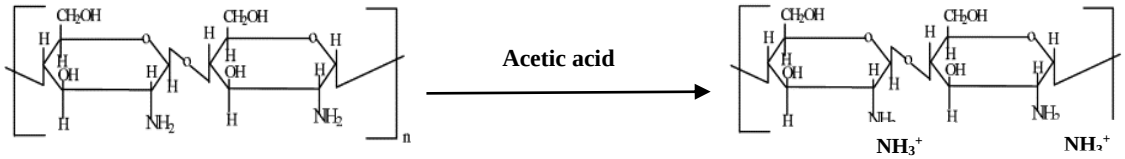
Chitosan is a natural biodegradable polymer obtained from the deacetylation of chitin and consist of β (1 → 4)-linked d-glucosamine and N-acetyl-d-glucosamine units (**Fig. 2.8**). Chitosan is the most abundant biopolymer next to cellulose in the world [103], and is receiving great interest in the encapsulation of active compounds due to its biocompatibility, low toxicity, and biodegradability [104], especially the unique cationic property when dissolved in acidic condition (1% acetic acid). The ionization of amino groups on the long chitosan chain made it a great candidate for crosslinking through ionic gelation and a promising drug delivery material [105].

(a) The formation of chitosan from chitin



Chitin **Chitosan**

(b) The ionization of amino group in chitosan chain at pH 5.6



Chitosan **Chitosan (cationic)**

(c) ionic crosslinking mechanism

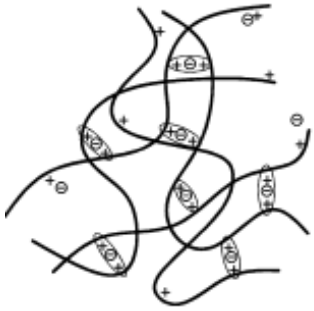


Fig. 2.8 Property of chitosan used as a cation
(Modified from reference [106]).

The studies on chitosan-based microcapsules included the investigations on titration direction [104] of chitosan to polyanions or in the opposite way and the mass ratio of chitosan to polyanion in encapsulation formulation [107]. Depending on the property of those anionic counterion, the hydrogels formed through ionic gelation are based on various crosslinking mechanisms. The next session introduced three typical combinations of chitosan-polyanion systems.

2.4.4.1 Chitosan-alginate

Alginate is a popular natural polymer obtained from the cell walls of brown seaweed and usually in sodium salt form, and is an anionic polysaccharide composed

of (1–4) linked b-D-mannuronate (M) and a-L-guluronate (G) units [108]. Alginate is biocompatible, biodegradable, readily available, inexpensive, and non-toxic. Due to the presence of carboxylate groups along the polymeric chains in aqueous phase, they are widely used as a vehicle for drugs, such as ampicillin [95], Ilex paraguariensis polyphenols [109]. The ionic interactions between alginate and chitosan are pH-dependent, and the repulsion of –COOH groups on chitosan chains is induced by the protonation in the low pH [103]. As a relatively hydrophobic material, alginate is not suggested in use of anthocyanin encapsulation.

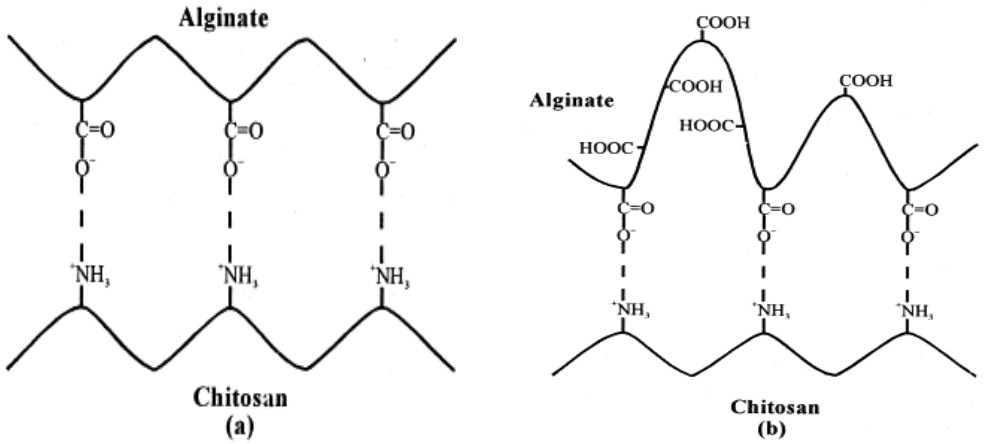


Fig. 2.9 Ionic interactions between alginate and chitosan: (a) pH 5.4; (b) pH 2.0. (Modified from references [103]).

2.4.4.2 Chitosan–TPP

Tripolyphosphate salt (TPP) is another popular small molecular weight counterion, and is a nontoxic multivalent anion. It can form gels by ionic interaction between positively charged amino groups of chitosan and negatively charged phosphate groups of TPP as long as pH<6.0 [110]. CH-TPP capsules have been widely investigated for encapsulating vitamin C [111] and tea polyphenols [113]. However, the use of this formulation on anthocyanin stabilizing has not been reported yet.

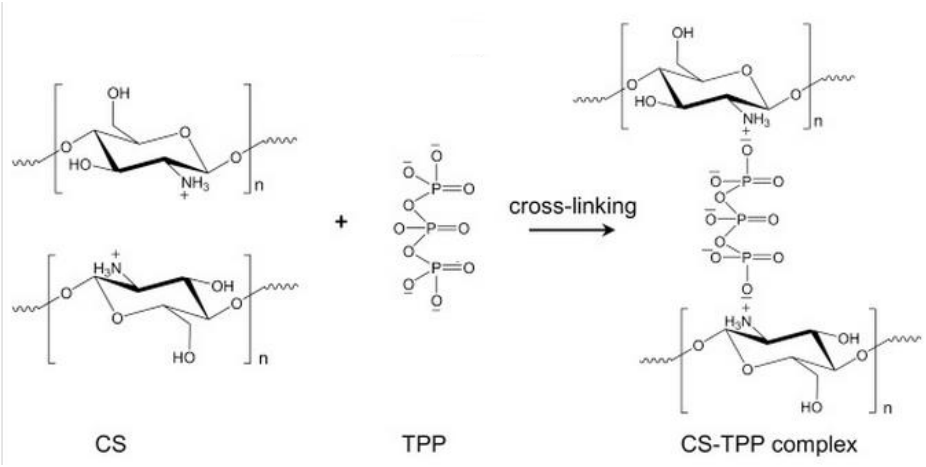


Fig. 2.10 Ionic interactions between tripolyphosphate salt and chitosan.

2.4.4.2 Chitosan–CNC

Cellulose nanocrystal (CNC) is a rod-like nanoparticle with high crystalline property, and isolated from cellulose fibers by acid hydrolysis with sulfuric acid. The diameter ranges from 5 to 20 nm and lengths of up to several hundred nanometers [106, 113]. The polyelectrolyte complexes formed by CNC and chitosan have exhibited well behaved mechanical properties as a vehicle for drug deliver with their high specific surface area, good mechanical strength, and biodegradability. Since the pKa of CNC is at pH 2.5, the sulfated anionic charges on the surface of CNC make it having strong attractions with other cations [114]. A recent study compared the biodegradable polymeric nanoparticles produced by alginate, chitosan and CNC to synthesize a double emulsion for poly(DL-Lactide-co-Glycolide) copolymer matrix, and found that the application of CNC induced a higher stabile oil/water interface for emulsion [115]. These findings helped form a hypothesis for using chitosan-CNC formulation to entrap anthocyanins with improved stability.

2.5 Conclusion

Anthocyanins as a promising natural pigment is highly unstable. It is important to develop strategies for maximal obtaining the amount of original anthocyanins and stabilizing their structures in the extracts during extraction and storage. In this research, first study emphasized on identifying optimal anthocyanin extraction conditions using both conventional solvent extraction and ultrasound-assisted extraction methods on three different anthocyanin rich fruit with different anthocyanin compositions. The second study evaluated chitosan based microencapsulation for stabilizing blueberry anthocyanin extracts and investigated the encapsulation efficiency of anthocyanins as affected by the type of anionic crosslinking agent (CNC and TPP), titration direction, and amount of loaded anthocyanins.

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Chapter 3

Optimization of Solvent and Ultrasound-assisted Extraction for Different Anthocyanin Rich Fruits and Their Effects on Anthocyanin Compositions

Abstract

Conventional solvent extraction (CE) and ultrasound-assisted extraction (UE) were optimized systematically for three fruit (blueberries, cherries, and red pear peels) having different anthocyanin compositions to pursue high recovery of polyphenols and anthocyanins with high antioxidant activity. The effects of the extraction methods and conditions on anthocyanin compositions remained in fruit extracts were also analyzed by HPLC. The optimal extraction conditions were identified as: 60% methanol, 50 ℃, for 1 hour using CE or 70% methanol, 30 ℃, for 20 min using UE for blueberries; 60% ethanol, 70 ℃, for 1 hour using CE or 80% ethanol, 30 ℃, for 20 min using UE for cherries; 60% methanol, 50 ℃, for 1 hour using CE or 60% ethanol, 30 ℃, for 60 min using UE for red pear peels. Ultrasound energy enhanced the higher retention of total monomeric anthocyanins and helped improving the solubility of polymeric anthocyanins in less polar solvent system for different fruit. HPLC analysis also revealed that extraction methods and conditions could alter the amount of certain individual anthocyanin compounds in fruit extracts, including the glycosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, or malvidin. Therefore, different conditions for CE and UE might be implemented for

fruit with different anthocyanin compositions for maximizing the recovery of anthocyanins.

3.1. Introduction

Anthocyanins are a group of polyphenolic flavonoids abundant in fruit, vegetables, and flowers, and contribute to their red, purple and blue colors. The potential health benefits of anthocyanins for preventing certain chronic diseases have been well studied [1]. Considering their non-toxicity and high biocompatibility to human body [2], anthocyanins drew increasing attentions from food manufacturers and researchers for their usage as natural pigments and antioxidants.

Anthocyanin is a glycoside derivative of an anthocyanidin (aglycon base in the form of flavylium), sugar moiety, and possible acylation from organic acids. There are six common anthocyanidins in nature with different substitution groups on ring B, including delphinidin (Dp), cyanidin (Cy), petunidin (Pt), pelargonidin (Pg), peonidin (Pn), and malvidin (Mv) [3]. The type and amount of individual anthocyanins vary among different anthocyanin rich fruit. For examples, blueberries contain all five anthocyanidins except Pg; cherries possess major Cy, and minor Pg and Pn [4]; and red pear peels mainly have Cy and minor Pn anthocyanins [5]. The applied extraction conditions (solvent type and concentration, pH, temperature, time, etc.) have been found directly impacting the chemical structure, concentration, and antioxidant activity of anthocyanins remained in extracts [6, 7]. Therefore, it is important to identify the optimal extraction conditions for fruit having different anthocyanins compositions in order to maximize the recovery of polyphenols and anthocyanins in the fruit extracts.

This study attempted to identify the optimal extraction conditions for two commonly used extraction methods, conventional solvent extraction (CE) and ultrasound-assisted extraction (UE). CE method as the most common method was widely applied to extract polyphenols and anthocyanins from various plant materials. The solvents commonly used in CE include methanol, ethanol, and acetone at the concentration of 60-80% for extracting complex structures of anthocyanins with different polarity [8]. Turkmen et al.[9] reported that the type of solvents used could greatly impact the extraction efficiency of polyphenols from black and mate teas. Weak acids, such as acetic acid and citric acid are usually added into the extraction solvents to avoid the breakage of aromatic acyl acid linkages and/or aliphatic dicarboxyl acyl groups in anthocyanins [10]. Extraction time and temperature have also been considered as critical factors for CE method [11, 12]. Higher temperature and longer time may be used to increase the amount of extracted anthocyanins. However, the drawbacks of higher temperature and longer time are the potential structure modifications and/or degradations of bioactive compounds in the extracts, thus resulting in lower extraction efficiency [7].

Ultrasound, an oscillating sound pressure wave with a frequency over 20 kHz, has been used to enhance the extraction efficiency by increasing the mass transfer between solvent and plant material [13, 14]. The ultrasound-assisted solvent extraction has the benefits of increasing mass transfer and improving solvent penetration, thus having potential for reducing chemical usage and extraction temperature, increasing extraction rate and yield, and saving cost [15]. A few studies have attempted to develop optimal UE conditions. However, in those previous studies, only one typical plant

material was evaluated, such as coconut shells [16], annatto seeds [17], wine lees [18], and pomegranate [19]. In addition, although many studies utilized high performance liquid chromatography (HPLC) to identify anthocyanin compositions in the fruit [20, 21, 22], few investigated the possible impacts from extraction methods and conditions on anthocyanin compositions for the given fruit.

Therefore, the objective of this study was to systematically investigate the optimal extraction conditions of CE and UE methods for maximizing the recovery of anthocyanins in rich fruit with different anthocyanin compositions (blueberries, cherries, and red pear peels) through two combined experimental designs (Taguchi design and completely randomized two-factorial design). In addition, the effect of the extraction methods and conditions on anthocyanin compositions in three tested fruit was investigated by HPLC. This study would provide new insights into the impact of extraction methods and conditions on anthocyanin compositions in different anthocyanin rich fruit, and give guidelines on the specific CE and UE conditions.

3.2. Materials and Methods

3.2.1. Fruit materials

Fresh blueberries (*Vaccinium Cyanococcus*), sweet cherries (*Prunus avium*), and red pears *(Pyrus)* were purchased from a local market (Corvallis, OR, USA). Fruit were selected for uniform size and similar maturity, and stored in a 4 ℃ cooler for extraction within two days.

3.2.2. Chemical reagents

The following chemical reagents were used for the experiments: Folin-Ciocalteu's phenol reagent and gallic acid from Sigma-Aldrich (St. Louis, MO, U.S.A.), acetic acid and L-ascorbic acid from Avantor Performance Materials (Center Valley, PA, U.S.A), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (95%) from Alfa Aesar (Ward Hill, MA, U.S.A). Hydrochloric acid, sodium acetate, potassium chloride, sodium carbonate, and trifluoroacetic acid (TFA) were obtained from EMD Chemicals (Gibbstown, NJ, U.S.A.). ACS grade methanol, ethanol and acetone from VWR (Radnor, PA, U.S.A.) were used for the extraction, and HPLC grade water and methanol from EMD Chemicals (Gibbstown, NJ, U.S.A.) were used for HPLC analysis. Anthocyanin standards including Cyanidin, Pelargonidin, Cyanidin-3-galactoside, Cyanidin-3-glucoside, Delphinidin-3-glucoside, Peonidin, and Petunidin-3-glucoside were purchased from PhytoLab (Secaucus, New Jersey, USA).

3.2.3. Preparation of fruit samples for extraction

Anthocyanins are located at the mature cell membrane of fruits. For blueberries and cherries, both flesh and peels contain high amount of anthocyanins so that whole fruits were used for the extraction. Whereas, for red pears, almost all anthocyanins are accumulated in the peels [23], thus only the peels were taken and used for the extraction.

Approximately 50 g of fruit samples were ground in a stainless blender (Waring Products, Torrington, CT, U.S.A.) with liquid nitrogen. Three grams of the finely ground fruit powders were collected for the following extraction steps [24].

3.2.4. Extraction procedures

Fruit powders and prepared solvents (combinations of different types and concentrations of solvents are shown in **Tables 3.1** and **3.2**) were mixed at a 1:10 solid to solvent ratio in 50 mL centrifuge tubes (VWR, PA, U.S.A.). The mixtures were immersed in a water bath (Precision, VA, U.S.A.) or a 185 W ultrasonic water bath (Branson B-220H, SmithKline Co., PA, U.S.A.), respectively, at given temperatures and times (**Tables 3.1** and **3.2**).

The obtained extracts were centrifuged (International Equipment, MA, U.S.A.) at 10,000 g for 10 min at 4 ℃ and filtrated through Whatman No.1 filter paper. Filtrates were evaporated through a vacuum rotary evaporator (Brinkmann Instruments, NY, U.S.A.) at 40 ℃ to remove residual organic solvent in 3 min. Evaporated extracts were then transferred into 25 mL volumetric flask, and filled to the mark with distilled (DI) water to obtain an appropriate dilution factor for measurements. Diluted extracts were stored at a -80 ℃ freezer (VWR, PA, U.S.A.) until analysis.

3.2.5. Analysis of total phenolics content (TPC)

TPC was determined using the Folin-Ciocalteau assay [25]. Briefly, 0.5 mL of diluted extract or 0.5 mL of 0.1, 0.3, 0.5, and 0.7 mg/mL gallic acid solution (used as standard) was mixed with 0.5 mL of Folin-Ciocalteau reagent and 7.5 mL of DI water, respectively. The control was 0.5 mL of DI water. Mixtures were vortexed at room temperature for 20 min, and then transferred into a 40 ℃ water bath. Three mL of 20% sodium carbonate (w/v) was added and the solution vortexed for another 20 min. Samples were immediately cooled to room temperature in an ice bath for 3 min, and the absorbance of samples and standards were measured at

765 nm using a Shimazu UV160U spectrometer (Shimazu Corp., Kyoto, Japan). Results are expressed as gallic acid equivalent (GAE) mg/g fresh material (FW).

3.2.6. *Analysis of total monomeric anthocyanin (TMA)*

TMA was determined using the pH differential method [6]. The aqueous extracts were appropriately diluted with 0.025 mol/L potassium chloride buffer (pH=1.0) and 0.4 mol/L sodium acetate buffer (pH=4.5), respectively. The mixture was homogenized at room temperature for 15 min. The absorbance difference between pH 1.0 and pH 4.5 for the diluted extracts was determined at both $\lambda_{vis\text{-}max}$ and 700 nm, respectively. TMA value was calculated using the following formula:

$$A = [(A_{\lambda vis\text{-}max} - A_{700nm})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700nm})_{pH\ 4.5}] \quad (1)$$

$$TMA = (A \times MW \times DF \times Ve \times 1000) / (\varepsilon \times 1 \times M) \quad (2)$$

where $\lambda_{vis\text{-}max}$ was the maximal wavelength where the absorbance peak showed the highest value within the visible range, MW was the molecular weight of cyanidin 3-glucoside (449.2 g/mol), DF was the dilution factor, Ve was the extract volume, ε was the molar extinction coefficient of cyanidin 3-glucoside (26,900 L/mol cm), and M was the mass of the fruit powders. The results were expressed as cyanidin 3-glucoside equivalent (Cy-3-Glu) mg/100 g FW.

3.2.7. Analysis of DPPH radical scavenging activity (DPPH)

The DPPH (2,2-diphenyl-1-picryhydrazyl) assay was used to measure free radical scavenging capacity of the phenolic compounds in extracts [26]. One mL of diluted extracts and 0.005, 0.01, 0.05, and 0.1 mg/mL of ascorbic acid solution (used as standard) were mixed with 3 mL of 9 mg DPPH agent solubilized in 100 mL of methanol. Mixtures were vortexed at room temperature and kept in the dark for 20

min. The absorbance of samples and standards was read at 517 nm using the same spectrometer as used for TPC, and the results were reported as ascorbic acid equivalents (AAE) mg/g FW.

3.2.8. Characterization of anthocyanins

3.2.8.1. Percent polymeric color (PPC)

PPC indicates the amount of polymeric anthocyanin compounds which are more stable against the environmental conditions in comparison with the monomeric anthocyanin. PPC was calculated as the polymeric color divided by color density [6], i.e., the ratio of polymerized anthocyanins in total anthocyanins.

$$\text{PPC} = (\text{polymeric color}/\text{color density}) \times 100\% \quad (3)$$

The polymeric color and color density were determined using the method of Cao et al [27]. Briefly, 0.2 mL of 0.2 g/mL potassium meta-bisulfite was added into 2.8 mL of diluted sample (bleached sample). And 0.2 mL of distilled water was added to 2.8 mL of diluted sample as the control sample. After equilibrating for 30 min at ambient conditions, the absorbance of the samples was evaluated at wavelength of 420 nm, 700 nm, and $\lambda_{vis\text{-}max}$, respectively.

Color density and polymeric color were all calculated using the formula:

$$[(A_{420nm}-A_{700nm}) + (A_{\lambda vis\text{-}max}-A_{700nm})] \times \text{dilution factor} \quad (4)$$

where the color density was tested on the control sample, and the polymeric color was test on the bleached sample using same formula.

3.2.8.2. HPLC characterization and quantitation

Anthocyanin compositions in blueberry, cherry and red pear peel extracts were determined using a Shimadzu liquid chromatograph system (Shimadzu,

Kyoto, Japan) monitored by a LC-10A VP control system equipped with two LC-10AS liquid chromatograph, one SPD-20A UV/VIS detector, and a remote controlling computer with Shimadzu EZStart 7.2.1 SP1 software. The procedures applied were modified on the method from Bordonaba et al. [28] Briefly, a 0.22 μm syringe was used to manually inject 10 μL of diluted samples into a C18 column (250 x 4.60 mm, 5 μm particle size) (Restek Co., PA, U.S.A). HPLC grade water was used to dilute extracts at the following ratios before the injection: blueberry (1:25 v:v), cherry (1:5 v:v), and red pear (no dilution). The two mobile phases used were (A) 2% acetic acid (v/v) in HPLC grade water, and (B) 2% TFA (v/v) in HPLC grade methanol. The gradient conditions were 0-10 min, 2-20% B; 10–20 min, 20 – 25% B; 20-25 min, 25-35% B; 25–35 min, 35-75% B; 35-45 min, 75% B. The total operation time was 45 min. The flow rate was set up at 1 mL/min and the column was held at room temperature (20-25 ^{o}C). Samples were thawed in a 4 ^{o}C refrigerator for 30 min prior to injection.

Eluted compounds from the fruit extracts were all detected at 540 nm. The presence and quantity of each anthocyanin were determined by comparing the peak area with external standards, Cy-3-gal, Cy-3-glu, and Cy-3-rut. Peak identification of compounds with no external standard were conducted by consulting previous literatures [4, 28, 29], and quantified using external standards that were closest to their chemical structure; Dp-3-gal, Dp-3-glu, and Dp-3-ara with Delphinidin-3-glucoside, Pt-3-gal, Pt-3-glu, and Pt-3-ara with Petunidin-3-glucoside, Pn-3-glu and Pn-3-ara with Peonidin and Mv-3-gal with cyanidin-3-galactoside and Mv-3-glu and Mv-3-ara with Cyanidin-3-glucoside. Validation of the method was conducted using spike

recoveries, in which a known amount of anthocyanin was added to a sample and the resulting concentration was determined. All spike recoveries fell within 100% ±5%.

3.2.9. Experimental design

To optimize the extraction conditions for each method, two experimental designs were consecutively applied in this study. Taguchi design was firstly applied to select the most important two contributing factors among the commonly applied three factors, assuming that there was no interaction between the factors. Taguchi design offered a simple and systematic approach to reduce the number of treatment combinations when multiple factors were considered, and also informed the contribution of individual treatment factor [30]. This information would be especially valuable for industrial or scale-up extraction process, considering less number of factors critically affecting quality characteristics of obtained extracts. For CE, the type of the organic solvent (methanol, ethanol and acetone), concentration of the solvent in 1% acetic acid solution (v/v/v) (60, 70 and 80%), and temperature (50, 60 and 70 ^{o}C) were widely investigated in the previous studies [12, 31]. The extracting time was applied with 1 hour for all combined extracting conditions. For UE, the treatment times (20, 40, and 60 min) were considered along with the solvent types (methanol, ethanol and acetone) and concentrations of the solvents in 1% acetic acid solution (v/v/v) (60, 70 and 80%) at temperature of 30 ± 0.5 ℃. The temperature effect was not considered in UE method based on our preliminary studies, showing that the combined UE and high temperature reduced anthocyanin contents in the extract, probably caused by

severe extraction condition along with the degradation of anthocyanin compounds (data not shown).

In Taguchi design, two parameters are used to determine the most contributing factors. The first parameter K_{ij} was the average value of each measured functional parameter in level j (j=1, 2, 3) of each factor i (i=A, B, C, D) and expressed as[32]:

$$K_{ij} = \frac{1}{N_i} \sum_{u=1}^{N_i} y_{i,j,u} \qquad (5)$$

where i represented the factor A, B and C; j represented the level 1, 2 and 3; N_i was the number of trials for each factor; and $y_{i,j}$ was the measured responses of factor i at level j. K_{ij} could explain how measured responses change with different levels of each treatment factor in the extraction conditions.

The second parameter, R_i, was the difference between the highest and lowest values of Kij to determine the most contributed factor, and expressed as:

$$R_i = (K_{ij})_{\max} - (K_{ij})_{\min} \qquad (6)$$

where $(K_{ij})_{max}$ and $(K_{ij})_{min}$ indicated the highest and lowest values of the measured values under each factor, respectively. The factor which had the highest R_i value was considered as the most contributing factor in the extraction conditions. The two mostly contributing factors for each extraction method were chosen by considering both the rank of R_i values for each factor and the factors showing significance ($P < 0.05$) based on ANOVA analysis.

Based upon the results obtained from Taguchi design, a 2x2 completely randomized factorial design was then applied to optimize the extraction conditions, in

which the possible interactions between the two selected factors was considered and high contents of TPC, TMA, DPPH, and PPC values in extracts were targeted. HPLC analysis was further conducted for investigating the effect of different extraction methods and conditions on the anthocyanin compositions in each fruit extract.

2.10. Statistical analysis

All experiments were conducted in triplicates. For a 2x2 completely randomized factorial design, the analysis of variance (ANOVA) was conducted to evaluate significant effect of the individual factor and their interactions. The multiple comparison *post-hoc* least significant difference test (LSD) was used to examine the difference among the levels of extraction factors at $P< 0.05$. ANOVA and LSD were performed using SAS 9.2 (SAS Institute Inc., NC, U.S.A.). Principal component analysis (PCA) was conducted to show classification of the individual anthocyanin in blueberry extracts (contained up to 14 identified compounds) in HPLC analysis and the relationship of the variables in the extraction conditions using XLSTAT from Addinsoft (New York, NY, U.S.A.).

3.3 Results and discussions

3.3.1. Two most contributing extraction factors based on the analysis of Taguchi design

Taguchi design successfully selected the most contributing two factors among the three treatment factors for each extraction method (**Tables 3.1** and **3.2**). For CE (**Table 3.1**), type of solvent (factor A) and temperature (factor C) were

identified as the two most contributing factors on TPC and TMA for all fruits and DPPH for red pear peels. Type of solvent (factor A) and solvent concentration (factor B) were most important for blueberries, and temperature (factor B) and solvent concentration (factor C) were most important for cherries. Further analysis through ANOVA (data not shown) provided a more convincing evidence for the significant effect of the type of solvent and temperature on the different measurements (marked as asterisk in **Table 3.1**). Hence, two treatment factors, the type of solvent and temperature, were selected for optimizing the conditions of CE method using a 2x2 completely randomized factorial design.

In respect to UE (**Table 3.2**), for blueberries, type of solvent (factor A) and concentration (factor B) appeared to be the most contributing two factors on both TPC and DPPH, whereas solvent concentration (factor B) and time (factor C) were for TMA. For cherries, type of solvent (factor A) and solvent concentration (factor B) were identified as the two most contributing factors on both TMA and DPPH, but solvent concentration (factor B) and time (factor C) were for TPC. For red pear peels, type of solvent (factor A) and time (factor C) were represented as two most contributing factors on TPC, TMA and DPPH. Through ANOVA analysis (data not shown), it was found that the solvent concentration was only significant ($P<0.05$) on TMA in blueberries, whereas solvent concentration and type of solvent significantly ($P<0.05$) affected TPC and TMA of cherries, respectively. In red pear peels, no significant factor was detected (marked as asterisk in **Table 3.2**). For following two-factorial design and analysis, therefore, the type and concentration of solvent were selected for blueberries and cherries, whereas type of solvent and extraction time

were chosen for red pear peels.

3.3.2. Optimal level of significant treatment factors identified from the 2x2 completely randomized factorial design

Two factors at three levels chosen from the Taguchi analysis were investigated using a 2x2 completely randomized factorial design to determine the optimal CE and UE conditions for blueberries, cherries, and red pear peels. In addition to TPC, TMA, and DPPH of the fruit extracts, PPC, indicating polymeric anthocyanins in the extract, was also measured to investigate the effect of extraction conditions on polymeric anthocyanin structures [33]. PPC has been previously analyzed for processed blueberries [34] and strawberry purees [35].

Based on ANOVA analysis (data not shown), no significant interactive effects between the treatment factors on TPC, TMA, DPPH, and PPC was observed in the three types of fruit extracts using either CE or UE method. Hence, only the mean values of each treatment level within the independent factor showing significant effect on the different measurements were reported based on *post-hoc* LSD results (**Fig. 3.1**). For CE-blueberries, methanol extraction significantly (P<0.05) enhanced TPC and TMA in comparison with ethanol and acetone, respectively. Similar to our finding, Deng et al.[36] also found that methanol extracts contain higher TPC in blueberry leaf extracts; It was proved that methanol is the most effective solvent for the highest phenolics recovery from hull of mangosteen (*Garcinia mangostana Linn*) in comparison with ethanol, acetone, isopropanol, ethyl acetate, and distilled water [37]. Previous study also indicated that acetone significantly (P<0.05) enhanced PPC in the extract,

probably because less polar polymeric anthocyanins was more readily to be extracted in the least polar solvent mixture containing acetone (polarity of the solvents is in the order of acetone < ethanol < methanol < water) [38]. Although most of the studies applied aqueous solution for extraction considering the high water solubility of anthocyanins. de Souza et al.[39] proposed a solvent with a combination with two organic solvent (methanol and acetone), in which their study found that it can enhance polyphenol recovery in the extract from Brazilian berries than the use of single organic solvent. For UE-blueberries, methanol extract showed significantly (P<0.05) higher TPC and DPPH than that of acetone and ethanol extracts. In addition, solvent concentration at 70% and 80% significantly (P<0.05) enhanced TMA in comparison with 60%. This result contradicted with the result using CE, in which more polar TMA was recovered from lower concentrations of organic solvent in the mixture with high polarity [40]. This difference might be due to the applied ultrasound energy, it facilitated the formation of stable polymeric anthocyanins extracted using polar solvent mixture, thus obtaining more TMA in the extract.

For CE-cherries, the extracts using ethanol or at 70 °C showed significantly (P<0.05) higher TPC, TMA, and DPPH than at other conditions. PPC was significantly (P<0.05) higher when extraction at 50 °C compared with at 60 °C and 70 °C, which indicated the higher temperature could dissociate polymeric anthocyanins. Pinelo et al.[41] also found that 50 °C provided the highest polyphenol recovery from grape byproducts. For UE-cherries, TPC was significantly (P<0.05) higher in methanol or ethanol extracts, whereas higher TMA was found in the least polar acetone extract. This result was also inconsistent with that polar TMA can be

more recovered in more polar solvent system [40]. Likewise, it was probably because the ultrasound energy dissociated polymeric anthocyanins extracted in less polar acetone solvent mixture, thus releasing more TMA in the extract.

For CE-red pear peels, methanol extracts showed significantly (P<0.05) higher TMA and DPPH than acetone extracts, whereas ethanol and acetone extracts had significantly (P<0.05) higher TPC. Interestingly, PPC was significantly (P<0.05) increased with extending extraction time in UE-red pear peels. This result was contradicted with other fruit using UE, in which TMA was enhanced by ultrasound energy. This difference could be related to the different cell wall structures among different fruit. The ultrasound energy could enhance the mass transfer between solvent and the red pear peels with firm cell wall structure and thick wax cuticle layer, thus assisting the extraction of polymeric anthocyanins without dissociating the anthocyanins. Hence, it might be concluded that the ultrasound assisted extraction is preferred for materials with firm cell wall structure for effectively disrupting the cells.

Based on the above results and the criteria used for selecting the optimal the extraction conditions (high extraction efficiency and lower level of solvent concentration, temperature and time for saving cost, time, and energy), the optimal extraction conditions were set as: blueberries, 60% methanol at 50 °C for 1 hour using CE and 70% methanol at 30 °C for 20 min using UE; cherries, 60% ethanol at 70 °C for 1 hour using CE and 80% ethanol at 30 °C for 20 min using UE; red pear peels, 60% methanol at 50 °C for 1 hour using CE and 60% ethanol at 30 °C for 1 hour using UE.

By comparing optimal CE and UE on extracting anthocyanins and polyphenols for each fruit on the basis of all four measured parameters (Data not shown here). It presented higher PPC could be obtained in the extracts of blueberry by using UE. For cherry extraction, higher TMA was remained in the extracts by UE methods. For extraction on red pear peels, although CE method improve the TPC value, while higher DPPH and PPC were found in the extracts produced from UE methods.

3.3.3. Effects of different extraction conditions on anthocyanin compositions

Anthocyanin compositional chromatogram for different fruit extracts are provided in **Supplementary Fig. 3.1**. Anthocyanin compositions varied significantly depending on the type of fruit, in which blueberries contained more various anthocyanidins than cherries and red pear peels. Specifically, blueberry extracts contained: (1) delphinidin-3-galactoside (Dp-3-gal), (2) delphinidin-3-glucoside (Dp-3-glu), (3) cyanidin-3-galactoside (Cy-3-gal), (4) delphinidin-3-arabinoside (Dp-3-ara), (5) cyanidin-3-glucoside (Cy-3-glu), (6) petunidin-3-galactoside (Pt-3-gal), (7) cyanidin-3-arabinoside (Cy-3-ara), (8) petunidin-3-glucoside (Pt-3-glu), (9) petunidin-3-arabinoside (Pt-3-ara), (10) peonidin-3-glucoside (Pn-3-glu), (11) malvidin-3-galactoside (Mv-3-gal), (12) peonidin-3-arabinoside (Pn-3-ara), (13) malvidin-3-glucoside (Mv-3-glu), and (14) malvidin-3-arabinosides (Mv-3-ara). Cherry extracts were composed of three anthocyanins: (1) Cy-3-glu, (2) cyanidin-3-rutinoside (Cy-3-rut), and (3) an unknown compound. Cy-3-rut was only found in cherry extracts. Red pear peel extracts contained two anthocyanins: (1) Cy-3-gal and (2) Cy-3-glu. These results are similar to those found by Barnes et al.[42], they identified 25 individual anthocyanins in blueberries with similar anthocyanidin

compositions. The presence of Cy-3-rut was also reported as a main compound in dark colored cherries by other researcher [43]. For red pears, Cy-3-gal was identified the major individual anthocyanins [44].

ANOVA results showed the effect of independent factors and their interactions on the anthocyanin compositions in different fruit extracts using CE or UE method (**Table 3.3**). For CE-blueberries, solvent types had significant (P<0.05) effect on quantities of 11 different anthocyanins except for Dp-3-glu, Pt-3-glu, and Pn-3-glu. Temperature had significant (P<0.05) effect on quantities of Dp-3-gal, Dp-3-ara, Cy-3-glu, Mv-3-gal, Pn-3-ara, Mv-3-glu, and Mv-3-ara. Significant (P<0.05) interactive effects between solvent type and temperature were found on Dp-3-gal, Cy-3-gal, Dp-3-ara, Cy-3-glu, Pn-3-glu, Mv-3-gal, Pn-3-ara, Mv-3-glu, and Mv-3-ara. For UE-blueberries, solvent types had significant (P<0.05) effect on 9 different anthocyanins, excluding Dp-3-glu, Cy-3-glu, Pt-3-gal, Cy-3-ara, and Pt-3-ara. Solvent concentrations significantly (P<0.05) affected Dp-3-gal, Dp-3-glu, Cy-3-gal, Pn-3-glu, Mv-3-gal, Pn-3-ara, and Mv-3-ara. Significant (P<0.05) interactive effects between solvent type and concentration were found on Cy-3-gal, Pt-3-ara, Pn-3-glu, Mv-3-gal, Pn-3-ara, Mv-3-glu, and Mv-3-ara. For CE-cherries, Cy-3-glu was significantly (P<0.05) affected by solvent type, whereas solvent type, temperature, and their interaction had significant (P<0.05) effect on Cy-3-rut. For UE-cherries, Cy-3-rut was significantly (P<0.05) affected by solvent type, concentration, and their interaction. For CE-red pear, solvent type had significant (P<0.05) effect on both Cy-3-glu and Cy-3-rut, whereas the significant (P<0.05) interaction between

solvent type and temperature was found on Cy-3-glu. For UE-red pear peels, only solvent type had significant (P<0.05) effect on Cy-3-gal. Hence, ANOVA analysis analysis demonstrated different susceptibility of individual anthocyanin compound to the extraction methods and conditions.

Multiple comparison *post-hoc* LSD test results are reported in **Table 3.4** to illustrate the effect of extraction methods and conditions (i.e., combined two levels of each factor) on the quantity of individual anthocyanins in blueberry, cherry, and red pear peel extracts. For CE-blueberries, Dp-3-gal, Cy-3-gal, Dp-3-ara, Cy-3-glu, Pn-3-glu, Mv-3-gal, Pn-3-ara, Mv-3-glu, and Mv-3-ara were significantly (P<0.05) higher when using acetone at 50 ℃, acetone at 50 ℃ and 70 ℃, methanol at 50 ℃, acetone at 50 ℃, acetone and methanol at 70 ℃, methanol at 70 ℃, acetone at all three temperatures and methanol at 70 ℃, or acetone at 50 ℃, respectively, in comparison with other combined levels of extractions. These results demonstrated that acetone promoted the extraction of Dp-3-gal, Cy-3-gal, Cy-3-glu, Pn-3-glu, Pn-3-ara, and Mv-3-ara, whereas methanol resulted in higher extraction efficiency on Dp-3-ara, Pn-3-glu, Mv-3-gal, Pn-3-ara and Mv-3-glu. For CE-cherries, Cy-3-rut was significantly (P<0.05) higher with methanol at 60 ℃, and for red pear peels Cy-3-gal was detected significantly (P<0.05) higher with ethanol at 50 ℃. These differences on the optimal extraction conditions might be related to the different polarity of each anthocyanin compound toward solvent. For UE-blueberries, Cy-3-gal, Pn-3-glu, Mv-3-gal, Pn-3-ara, Mv-3-glu, and Mv-3-ara were significantly (P<0.05) higher when extracted using 60 to 80% acetone, 70 and 80% methanol, 70% acetone, 60% methanol, 70% acetone, and 70% methanol, respectively, in comparison with other combined levels of

extractions. Acetone enhanced the extraction of Cy-3-gal, Mv-3-gal, and Mv-3-glu, whereas methanol was more suitable for extracting Pn-3-glu, Pn-3-ara, and Mv-3-ara. The 70% acetone and ethanol extracted higher Mv-3-gal and Mv-3-glu. For UE-cherries, 60% ethanol or 60 and 70% methanol significantly ($P<0.05$) increased Cy-3-rut, compared with other treatments. These results demonstrated that the extracting condition selectively altered anthocyanin compositions in the extract, thus the specific extraction conditions could be selected based on the individual anthocyanin compound of interest.

To further interpret the correlation of anthocyanin compositions with the extraction conditions we used, PCA for blueberry extracts showed that specific extraction conditions favor different groups of anthocyanin compounds (**Fig. 3.2**). The first two factors in PCA accounted for 66.43% of the total variance (F1=41.64%, F2=24.79%). Specifically, along F1 showed two groups, one along the negative axis and the other along the positive axis. This was characterized by a greater number of anthocyanins and occurred because UE produced a lower number of overall anthocyanins in the resulting extracts. Of these two main groups, CE-1, CE-2, CE-3 and CE-5 were characterized by Cy-3-glu, Mv-3-ara, Pn-3-ara, and Pt-3-ara, and CE-9, UE-1, UE-2 and UE-3 were characterized by Cy-3-ara, Dp-3-ara, Mv-3-gal, Mv-3-glu and Pt-3-glu, respectively. CE-7, UE-4, UE-5, UE-6 and UE-9 were not characterized by any specific groupings of anthocyanins, but were found to contain similar amounts of anthocyanins as they were found on the PCA graph in between the anthocyanin vectors. CE-4, CE-6 and CE-8 contained lower concentrations of the anthocyanins due to their closer

proximity to the (0, 0) point in the PCA figure. UE-7 was characterized by Dp-3-glu and Pt-3-gal. UE-8 was characterized by Pn-3-glu. Therefore, it can be seen that depending on the anthocyanin(s) of interest, there were different extraction methods and conditions that may be preferred over others.

3.4. Conclusion

Both conventional solvent extraction and ultrasound-assisted extraction methods were optimized to enhance the recovery of polyphenols, anthocyanins, and antioxidant activity in the extracts from blueberries, cherries, and red pear peels containing different anthocyanin compositions. The extracting conditions for each method were systematically optimized through the Taguchi analysis and 2x2 completely randomized two-factorial design. It was found that UE method enhanced the formation of polymeric anthocyanins in blueberry and red pear peel extracts and increased total monomeric anthocyanin content in cherry extracts. The ultrasound assistance might be more suitable for materials with firm cell wall structure for effective cell disruptions, thus allowing the use of low concentration of solvent and lower temperature for anthocyanin extraction. HPLC analysis revealed that extraction methods and conditions altered the specific anthocyanin compositions in fruit extracts. Depending on the individual anthocyanins of interest, different extraction methods and conditions can be applied. Other extraction assistance technologies, such as microwave and high hydrostatic pressure may be evaluated to examine the optimal extraction method and conditions on different anthocyanin rich fruits in future work. Additionally other types of anthocyanin rich fruits with different anthocyanin

compounds may also be investigated using the developed optimal extraction conditions to compare with the results obtained from this study.

Acknowledgements

The authors would like to thank the USDA AFRI Organic Research Education Initiative for partially funding this project (Project No. 2008-01237).

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Table 3.1 Contribution of each treatment factor on total phenolic content (TPC), total monomeric anthocyanin (TMA), and DPPH radical scavenging activity of blueberries, cherries, and red pear peels using conventional solvent extraction (CE) method

				Type of fruits								
	Factors			Blueberries			Cherries			Red pear peels		
No.	**A**	**B**	**C**	TPC	TMA	DPPH	TPC	TMA	DPPH	TPC	TMA	DPPH
1	Methanol	60	50	2.74	37.96	4.84	1.28	4.12	0.80	1.55	2.23	5.73
2	Methanol	70	60	2.77	46.29	4.61	1.39	6.17	0.90	1.56	1.59	5.95
3	Methanol	80	70	3.05	49.83	4.22	1.31	4.81	0.86	2.14	3.42	6.90
4	Ethanol	60	60	3.28	40.55	2.24	1.46	5.90	0.89	1.16	0.92	5.56
5	Ethanol	70	70	3.51	55.59	4.97	1.46	4.58	0.87	2.00	2.40	6.57
6	Ethanol	80	50	3.01	60.73	1.02	1.42	4.00	0.82	1.92	2.13	6.15
7	Acetone	60	70	3.64	63.09	1.53	1.39	3.10	0.88	2.52	3.01	7.52
8	Acetone	70	50	3.16	53.01	4.68	1.37	2.69	0.84	1.94	1.74	6.33
9	Acetone	80	60	3.08	52.99	5.17	1.40	3.59	0.81	1.85	1.47	6.65
Factor	levels											
A	K_{A1}			2.86^{b}	44.69^{b}	4.55^{a}	1.33^{a}	5.03^{a}	0.86^{a}	1.75^{b}	2.41^{a}	6.19^{b}
	K_{A2}			3.27^{a}	52.29^{ab}	2.74^{b}	1.45^{a}	4.83^{a}	0.86^{a}	1.69^{b}	1.82^{b}	6.09^{b}
	K_{A3}			3.29^{a}	56.36^{a}	3.79^{ab}	1.39^{a}	3.12^{b}	0.84^{a}	2.10^{a}	2.07^{ab}	6.83^{a}
	R_A[+]			0.44	11.67	1.81	0.12	1.91	0.01	0.41	0.60	0.74
B	K_{B1}			3.22^{a}	47.20^{a}	2.87^{b}	1.38^{a}	4.37^{a}	0.86^{a}	1.75^{b}	2.05^{a}	6.27^{a}
	K_{B2}			3.15^{a}	51.63^{a}	4.75^{a}	1.41^{a}	4.48^{a}	0.87^{a}	1.83^{ab}	1.91^{a}	6.28^{a}
	K_{B3}			3.05^{a}	54.52^{a}	3.47^{ab}	1.37^{a}	4.13^{a}	0.83^{a}	1.97^{a}	2.34^{a}	6.57^{a}
	R_B			0.17	7.31	1.88	0.03	0.35	0.04	0.22	0.43	0.30
C	K_{C1}			2.97^{b}	50.57^{a}	3.51^{a}	1.36^{a}	3.60^{b}	0.82^{b}	1.80^{b}	2.03^{b}	6.07^{b}
	K_{C2}			3.04^{b}	46.61^{a}	4.01^{a}	1.42^{a}	5.22^{a}	0.87^{a}	1.52^{c}	1.33^{c}	6.05^{b}
	K_{C3}			3.40^{a}	56.17^{a}	3.57^{a}	1.38^{a}	4.16^{ab}	0.87^{a}	2.22^{a}	2.94^{a}	6.99^{a}
	R_C			0.43	9.56	0.49	0.06	1.62	0.05	0.70	1.62	0.94
	Rank[++]			A(*)>C(*)>B	A>C>B	B>A>C	A>C>B	A(*)>C>B	C(*)>B>A	C(*)>A(*)>B	C(*)>A>B	C(*)>A(*)>B
Two most contributing factors				Factor A and C			Factor A and C			Factor A and C		

Factor A, B, and C represent types of solvent, concentration (%) of solvent in 1% acetic acid, and temperature (℃), respectively.

Units for TPC, TMA, and DPPH value were gallic acid equivalent mg/g fresh material, cyanidin-3-glucoside equivalent mg/100 g fresh material, ascorbic acid equivalent mg/g fresh material, respectively.

[+]Kij were the average values of each measured parameter from nine treatment set in level j (j=1, 2, 3) of each factor i (i=A, B or C). The same lowercase letter above Kij of each measured parameter indicated no significant difference detected by LSD test among levels for the same factor ($P>0.05$).

[++] R_A, R_B, and Rc were the differences between the highest and lowest values of Kij within the same factor i

[+++] Rank was based on the largest to smallest order of R_A, R_B, and Rc value, the factor with asterisk meant significance detected by ANOVA analysis ($P<0.05$)

[++++] The two most contributing factors were selected based on the significance and rank order of Ri.

Table 3.2 Contribution of each treatment factor on total phenolic content (TPC), total monomeric anthocyanin (TMA), and DPPH radical scavenging activity of blueberries, cherries, and red pear peels using ultrasound-assisted extraction (UE) method

				Type of fruits								
	Factors			Blueberries			Cherries			Red pear peels		
No.	**A**	**B**	**C**	TPC	TMA	DPPH	TPC	TMA	DPPH	TPC	TMA	DPPH
1	Methanol	60	20	2.04	32.42	4.75	1.63	7.54	0.91	1.43	0.62	5.46
2	Methanol	70	40	2.45	57.01	4.76	1.44	6.86	0.86	1.45	0.93	5.85
3	Methanol	80	60	2.62	54.46	4.19	1.44	7.04	0.91	1.50	2.48	5.67
4	Ethanol	60	40	3.00	44.08	2.24	1.56	8.68	0.89	1.48	0.65	5.71
5	Ethanol	70	60	2.04	45.83	4.85	1.37	5.18	0.84	1.73	0.78	5.76
6	Ethanol	80	20	2.48	46.61	1.02	1.44	6.62	0.88	1.53	0.48	5.85
7	Acetone	60	40	3.46	45.33	1.50	1.55	4.55	0.88	1.75	0.82	5.77
8	Acetone	70	20	2.99	48.70	4.60	1.47	5.40	0.88	1.56	0.79	5.51
9	Acetone	80	60	2.47	56.36	5.49	1.34	4.27	0.84	1.70	1.15	5.53
Factor	levels											
A	K_{A1}			2.37^{a}	47.96^{a}	4.57^{a}	1.50^{a}	7.14^{a}	0.89^{a}	1.46^{b}	1.34^{a}	5.66^{a}
	K_{A2}			2.51^{a}	45.51^{a}	2.70^{b}	1.46^{a}	6.83^{a}	0.87^{ab}	1.58^{ab}	0.63^{a}	5.78^{a}
	K_{A3}			2.97^{a}	50.13^{a}	3.86^{ab}	1.45^{a}	4.74^{b}	0.86^{b}	1.67^{a}	0.92^{a}	5.60^{a}
	$R_A{}^{+}$			0.60	4.63	1.86	0.05	2.40	0.03	0.21	0.71	0.17
B	K_{B1}			2.84^{a}	40.61^{b}	2.83^{b}	1.58^{a}	6.92^{a}	0.89^{a}	1.55^{a}	0.70^{a}	5.65^{a}
	K_{B2}			2.49^{a}	50.51^{a}	4.74^{a}	1.43^{b}	5.81^{a}	0.86^{b}	1.58^{a}	0.83^{a}	5.71^{a}
	K_{B3}			2.53^{a}	52.48^{a}	3.57^{ab}	1.41^{b}	5.97^{a}	0.88^{ab}	1.58^{a}	1.37^{a}	5.68^{a}
	R_B			0.35	11.87	1.91	0.17	1.11	0.03	0.03	0.67	0.06
C	K_{C1}			2.50^{a}	42.58^{b}	3.46^{a}	1.51^{a}	6.52^{a}	0.89^{a}	1.51^{b}	0.63^{a}	5.61^{a}
	K_{C2}			2.64^{a}	52.48^{a}	4.16^{a}	1.45^{a}	6.60^{a}	0.86^{a}	1.54^{ab}	0.91^{a}	5.70^{a}
	K_{C3}			2.71^{a}	48.54^{ab}	3.51^{a}	1.46^{a}	5.59^{a}	0.88^{a}	1.66^{a}	1.36^{a}	5.74^{a}
	R_C			0.20	9.91	0.71	0.06	1.01	0.03	0.15	0.73	0.13
Rank^{++}				A>B>C	B(*)>C>A	B>A>C	B(*)>C>A	A(*)>B>C	A>B>C	A>C>B	C>A>B	A>C>B
Two mostly contributing factors				Factor A and B			Factor A and B			Factor A and C		

Factor A, B, and C represented the type of solvent, concentration (%) of solvent in 1% acetic acid, and time (min), respectively.

Units for each TPC, TMA, and DPPH value were gallic acid equivalent mg/g fresh material, cyanidin-3-glucoside equivalent mg/100g fresh material, ascorbic acid equivalent mg/g fresh material, respectively.

$^{+}$Kij were the average values of each measured parameter from nine treatment sets in level j (j=1, 2, 3) of each factor i (i=A, B or C). The same lowercase letter above Kij of each measured parameters indicated no significant difference detected by LSD test among levels for the same factor (P>0.05).

$^{++}$ R_A, R_B, and Rc were the difference between the highest and lowest values of Kij within the same factor i (i= A, B or C).

$^{+++}$ Rank was based on the largest to smallest order of R_A, R_B, and Rc value, the factor with asterisk meant significance detected by ANOVA analysis ($P<0.05$)

$^{++++}$ The two most contributing factors were selected based on the significance and rank order of Ri.

Table 3.3 Analysis of variance (ANOVA) results (p=0.05) for analyzing the effect of independence or interactions among treatment factors on different anthocyanin compounds of blueberry, cherry, and red pear peel extracts by using conventional solvent extraction (CE) and ultrasound-assisted extraction (UE) methods

(A) Blueberries

CE method

Type of anthocyanin[+]	Dp-3-gal	Dp-3-glu	Cy-3-gal	Dp-3-ara	Cy-3-glu	Pt-3-gal	Cy-3-ara	Pt-3-glu	Pt-3-ara	Pn-3-glu	Mv-3-gal	Pn-3-ara	Mv-3-glu	Mv-3-ara
Linear terms														
Solvent (SV)	0.0009	0.2189	0.0009	0.0001	0.0001	0.0003	0.0053	0.3054	0.0001	0.5888	0.0001	0.0001	0.0001	0.0001
Temperature (TP)	0.0190	0.5242	0.1601	0.0009	0.0002	0.7101	0.7759	0.5611	0.7703	0.2679	0.0001	0.0176	0.0001	0.0026
Interaction terms														
SV x TP	0.0004	0.8496	0.0013	0.0006	0.0001	0.3049	0.7807	0.4239	0.7553	0.0419	0.0001	0.0001	0.0001	0.0001

UE method

Linear terms														
Solvent (SV)	0.0001	0.4233	0.0001	0.0001	0.4823	0.5795	0.3896	0.0474	0.2076	0.0001	0.0001	0.0001	0.0001	0.006
Concentration (CT)	0.0311	0.0261	0.0058	0.1089	0.2817	0.1697	0.3440	0.2059	0.0967	0.0001	0.0001	0.0007	0.1087	0.0011
Interaction terms														
SV x CT	0.1438	0.0696	0.0005	0.3592	0.0648	0.6558	0.2550	0.8662	0.0220	0.0001	0.0001	0.0001	0.0001	0.0019

(B) Cherries

CE method

Type of anthocyanin	Cy-3-glu	Cy-3-rut
Linear terms		
Solvent (SV)	0.0282	0.0001
Temperature (TP)	0.0712	0.0001
Interaction terms		
SV x TP	0.8122	0.0007

UE method

Type of anthocyanin	Cy-3-glu	Cy-3-rut
Linear terms		
Solvent (SV)	0.1803	0.0001
Concentration (CT)	0.5852	0.0048
Interaction terms		
SV x CT	0.0792	0.0001

(C) Red pear peels

CE method

Type of anthocyanin	Cy-3-gal	Cy-3-glu
Linear terms		
Solvent (SV)	0.0068	0.0466
Temperature (TP)	0.1878	0.1946
Interaction terms		
SV x TP	0.0102	0.5594

UE method

Type of anthocyanin	Cy-3-gal
Linear terms	
Solvent (SV)	0.0010
Time (TM)	0.8764
Interaction terms	
SV x TM	0.4917

[+] Delphinidin-3-galactoside = Dp-3-gal; Delphinidin-3-glucoside = Dp-3-glu; Cyanidin-3-galactoside = Cy-3-gal; Delphinidin-3-arabinoside = Dp-3-ara; Cyanidin-3-glucoside = Cy-3-glu; Petunidin-3-galactoside = Pt-3-gal; Cyanidin-3-arabinoside = Cy-3-ara; Petunidin-3-glucoside = Pt-3-glu; Petunidin-3-arabinoside =Pt-3-ara; Peonidin-3-glucoside = Pn-3-glu; Malvidin-3-galactoside = Mv-3-gal; Peonidin-3-arabinoside = Pn-3-ara; Malvidin-3-glucoside = Mv-3-glu; Malvidin-3-arabinosides = Mv-3-ara.

Table 3.4 The effect of different extraction conditions on the individual anthocyanin compounds in blueberry, cherry, and red pear peel extracts by conventional solvent extraction (CE) and ultrasound-assisted extraction (UE) methods using multiple comparison *post-hoc* least significant difference (LSD) test

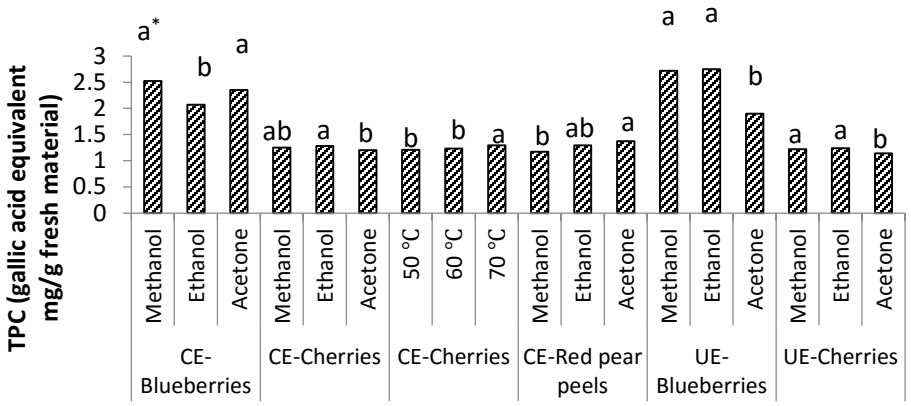
[A] Blueberry											
Factors		CE (μg/g FW)									
Solvent	Temp (℃)	**Label**	Dp-3-gal	Cy-3-gal	Dp-3-ara	Cy-3-glu	Pn-3-glu	Mv-3-gal	Pn-3-ara	Mv-3-glu	Mv-3-ara
Acetone	50	**CE-1**	109.96 a	147.08 a	13.46 d	170.19 a	28.00 ab	45.15 c	507.22 a	23.43 d	123.11 a
	60	**CE-2**	86.47 bc	131.39 ab	ND*	133.28 b	12.89 b	30.95 g	477.17 a	20.39 g	98.10 f
	70	**CE-3**	92.32 bc	145.67 a	ND	147.47 ab	52.31 a	25.97 h	547.34 a	26.14 b	114.53 b
Ethanol	50	**CE-4**	64.69 de	95.69 cd	ND	101.03 c	42.68 ab	182.74 b	12.14 d	16.68 h	85.01 g
	60	**CE-5**	86.26 bc	131.09 ab	ND	142.16 b	25.04 ab	36.44 f	463.19 ab	23.39 e	99.94 e
	70	**CE-6**	79.23 cd	115.60 bc	59.77 b	21.73 d	ND	39.64 d	359.88 bc	23.02 f	101.53 d
Methanol	50	**CE-7**	99.45 ab	136.89 ab	80.10 a	28.16 d	18.99 ab	36.74 e	468.06 a	26.11 c	113.44 c
	60	**CE-8**	60.48 e	83.83 d	44.80 c	15.79 d	28.61 ab	5.17 h	274.97 c	12.30 i	61.30 h
	70	**CE-9**	85.40 bc	121.35 b	62.29 b	22.15 d	53.72 a	223.63 a	29.65 d	92.81 a	ND
Factors		UE (μg/g FW)									
Solvent	Con (%)	**Label**	Cy-3-gal	Pn-3-glu	Mv-3-gal	Pn-3-ara	Mv-3-glu	Mv-3-ara			
Acetone	60	**UE-1**	124.40 a	43.34 bc	206.95 c	21.07 c	92.35 c	ND			
	70	**UE-2**	137.33 a	59.10 b	263.88 a	34.72 c	112.72 a	ND			
	80	**UE-3**	125.53 a	ND	235.97 b	27.32 c	100.34 b	ND			
Ethanol	60	**UE-4**	67.28 b	26.22 c	158.09 e	18.11 c	62.04 e	ND			
	70	**UE-5**	62.16 b	27.82 c	161.44 d	13.78 c	66.95 d	ND			
	80	**UE-6**	63.65 b	12.40 c	18.63 f	97.50 b	ND	ND			
Methanol	60	**UE-7**	ND	11.77 c	19.59 ef	278.42 a	12.28 g	ND			
	70	**UE-8**	81.94 b	320.65 a	15.43 g	114.27 b	ND	56.62 a			
	80	**UE-9**	63.11 b	256.16 a	4.28 h	18.80 c	56.16 f	ND			

Table 3.4 (Continued)

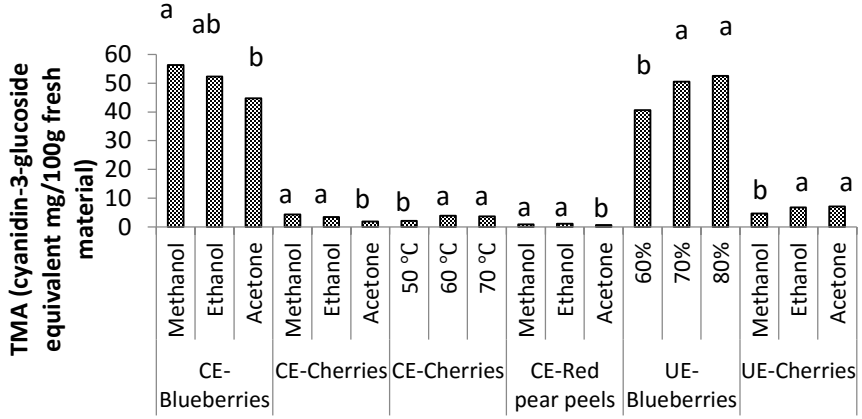
[B] Cherry								**[C] Red pear peel**						
Factors		CE (μg/g FW)		Factors		UE (μg/g FW)		Factors		CE (μg/g FW)		Factors		UE
Solvent	Temp (℃)	**Label**	Cy-3-rut	Solvent	Con (%)	**Label**	Cy-3-glu	Solvent	Temp (℃)	**Label**	Cy-3-gal	Solvent	Time (min)	**Label**
Acetone	50	**CE-1**	1.49 g	Acetone	60	**UE-1**	3.50 e	Acetone	50	**CE-1**	4.09 bcd	Acetone	20	**UE-1**
	60	**CE-2**	4.96 ef		70	**UE-2**	7.79 c		60	**CE-2**	6.60 ab		40	**UE-2**
	70	**CE-3**	6.00 de		80	**UE-3**	7.32 cd		70	**CE-3**	5.50 bcd		60	**UE-3**
Ethanol	50	**CE-4**	4.14 f	Ethanol	60	**UE-4**	14.98 a	Ethanol	50	**CE-4**	8.57 a	Ethanol	20	**UE-4**
	60	**CE-5**	9.92 b		70	**UE-5**	11.70 b		60	**CE-5**	6.39 abc		40	**UE-5**
	70	**CE-6**	7.91 c		80	**UE-6**	11.39 b		70	**CE-6**	3.95 cd		60	**UE-6**
Methanol	50	**CE-7**	6.60 d	Methanol	60	**UE-7**	15.53 a	Methanol	50	**CE-7**	4.31 bcd	Methanol	20	**UE-7**
	60	**CE-8**	12.06 a		70	**UE-8**	14.59 a		60	**CE-8**	3.41 d		40	**UE-8**
	70	**CE-9**	9.26 b		80	**UE-9**	10.17 bc		70	**CE-9**	4.04 cd		60	**UE-9**

* Columns with post-hoc LSD test (*) were only illustrated for fruits and treatment factors showing the significant effect in ANOVA results (shown in Table 3); The same letter behind each bar within each extraction method and the type of fruits were not significantly different (P>0.05) based on LSD test.

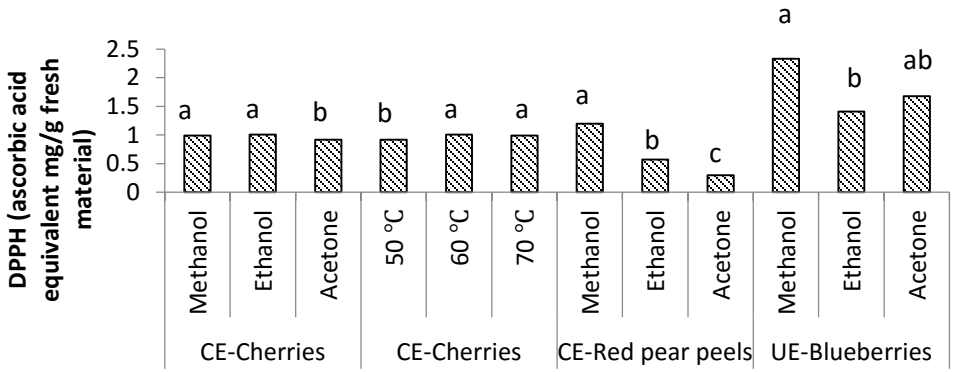
[A] Total phenolic content



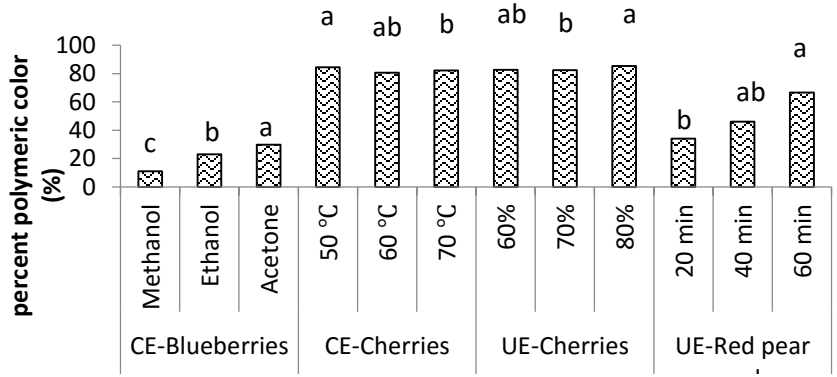
[B] Total monomeric anthocyanin



[C] DPPH radical scavenging capacity



[D] Percent polymeric color



(Continued for the Figure in the last page)

Fig. 3.1 The effect of main treatment factor on total phenolic content (TPC) [A], total monomeric anthocyanin (TMA) [B], DPPH radical scavenging activity [C], and percent polymeric color (%) [D] of three types of fruit extracts using either conventional solvent extraction (CE) or ultrasound-assisted extraction (UE) based on analysis of variance (ANOVA) and multiple comparison *post-hoc* least significant difference (LSD). Figures with *post-hoc* LSD test (*) were only illustrated for fruits and treatment factors showing the significant effect in ANOVA results (data not shown); The same lowercase letter above each bar within each extraction method and the type of fruits were not significantly different ($P>0.05$) based on LSD test.

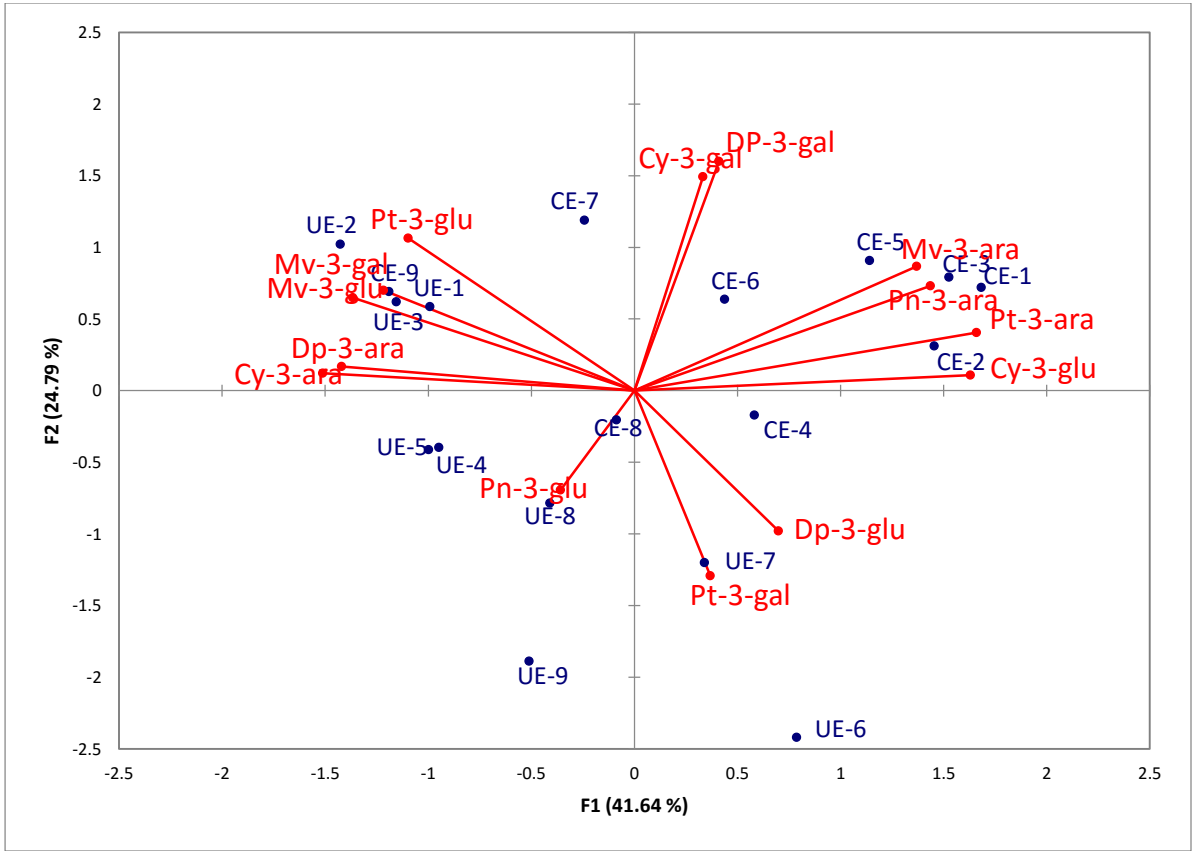
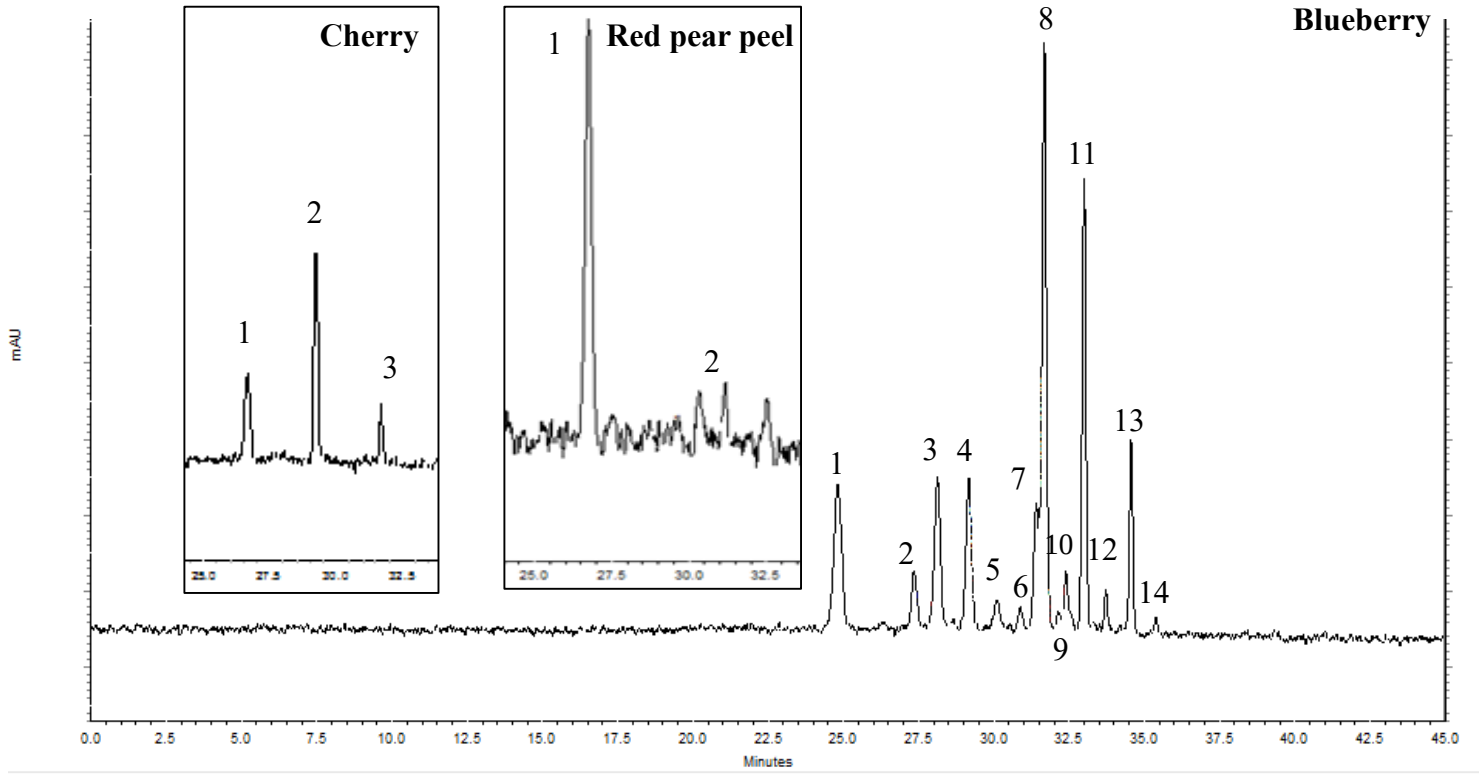


Fig. 3. 2 PCA for blueberry extraction conditions on individual anthocyanins.

Delphinidin-3-galactoside = Dp-3-gal; Delphinidin-3-glucoside = Dp-3-glu; Cyanidin-3-galactoside = Cy-3-gal; Delphinidin-3-arabinoside = Dp-3-ara; Cyanidin-3-glucoside = Cy-3-glu; Petunidin-3-galactoside = Pt-3-gal; Cyanidin-3-arabinoside = Cy-3-ara; Petunidin-3-glucoside = Pt-3-glu; Petunidin-3-arabinoside =Pt-3-ara; Peonidin-3-glucoside = Pn-3-glu; Malvidin-3-galactoside = Mv-3-gal; Peonidin-3-arabinoside = Pn-3-ara; Malvidin-3-glucoside = Mv-3-glu; Malvidin-3-arabinosides = Mv-3-ara.

*The specific extraction conditions of blueberry label as CE-1 to CE-9 and UE-1 to UE-9 could be found in Table 4.



Supplemental Fig. 3.1 Anthocyanin profiles in blueberry, cherry, and red pear peel extracts analyzed by HPLC
Blueberries contain (1) Dp-3-gal, (2) Dp-3-glu, (3) Cy-3-gal, (4) Dp-3-ara, (5) Cy-3-glu, (6) Pt-3-gal, (7) Cy-3-ara, (8) Pt-3-glu, (9) Pt-3-ara, (10) Pn-3-glu, (11) Mv-3-gal, (12) Pn-3-ara, (13) Mv-3-glu, and (14) Mv-3-ara; Cherries mainly contain (1) Cy-3-glu, (2) Cy-3-rut, and (3) unknown; Red pear peels mainly contain (1) Cy-3-gal, and (2) Cy-3-glu.

Chapter 4

Investigation of Sodium Tripolyphosphate (TPP) and Cellulose Nanocrystals (CNC) as Anionic Cross-Linking Agents for the Microencapsulation of Blueberry Anthocyanins

Abstract

Anthocyanins (ACN) are natural food colorants with high antioxidant capacity, but are sensitive to pH, temperature, light, and oxygen during processing and storage. Chitosan (CH) based microencapsulation was applied to encapsulate blueberry anthocyanin extracts (BB ACN) to enhancing their stability against surrounding conditions. Two different anionic crosslinking agents (cellulose nanocrystal (CNC) and sodium tripolyphosphate (TPP)) were selected to form microcapsules through ionic gelation with CH. The yield of microcapsules (YOM), total monomeric anthocyanin (TMA) recovery, particle characteristics, total phenolic content (TPC), and DPPH radical scavenging activity of the collected microcapsules or remained supernatants after centrifugation were investigated by considering various influencing factors, including titration direction, concentration of anionic crosslinking agents, and the amount of loaded BB ACN. Results showed that YOM (~6.9-8.1 g) and TMA recovery (~85-94%) of BB-CH-CNC microcapsules were significantly (P<0.05) higher than those of BB-CH-TPP microcapsules (~0.3-0.7 g and ~33-42%, respectively). When using CNC as a carrier of BB ACN extracts

titrated into CH bath, the low polydispersity index (PDI <0.3) was achieved with larger particle size than adverse direction (BB – CH titrate into CNC bath). But TMA recovery showed no significant difference between two different titration directions within the same crosslinking agent. Increase on YOM, TMA recovery and less free TPC and DPPH of supernatant were then found when the concentration of crosslinking agent was reached at 1.0% (w/v). BB ACN distribution in the microcapsules (on surfaces, bound with matrix, and in core) is dependent on the amount of loaded BB ACN, but BB-CH-CNC microcapsules may transfer the majority of TMA in the core (up to 48%), whereas BB-CH-TPP microcapsules had TMA increasingly bound in the matrix (more than 95%). The light microscopic images illustrated that BB-CH-CNC microcapsules successfully carried BB ACN with spherical morphology, whereas BB-CH-TPP microcapsules showed irregular morphology and holes within crosslinked joints of microcapsule clusters. This study provided new insights on using CNC as an anionic crosslinking agent for CH based microencapsulation to form rigid and stable microcapsules for loading ACN.

4.1 Introduction

Anthocyanins (ACN) are natural food pigments with broad color range (from bright red to purple and dark blue) existing in various plant materials. They are flavonoids, and occur as glycoside forms of anthocyanidin (aglycone) possessing high antioxidant capacity [1]. Blueberries contain high ACN content (25-495 mg/100g FW) [2] with up to 25 different individual ACN, including different species of anthocyanidin (delphinidin, cyanidin, peonidin, petunidin and malvidin) [3].

ACN is non-toxic and highly biocompatible for human body, and is considered a promising substitute for synthetic colorant. However, its stability in aqueous media depends on its chemical structure and concentration of ACN and/or surrounding conditions, as well as pH, light, oxygen,

or temperature. Red color of cationic ACN flavylium is able to be degraded into colorless carbinol pseudo-base and formed yellowish chalcone when pH is over 2.5. These can further be broken down into small colorless aldehydes or phenolic acids under various surrounding conditions [4–6]. Therefore, demands on effective stabilization technique are needed, aiming to preserve ACN from the external conditions and thus extending its storability for further applications.

Microencapsulation technique forms a shell/wall outside the bioactive core compounds, thus protecting them from surrounding conditions and/or controlling the delivery and/or release of the core compounds in food or drug system, for particles that are in less than 1000 μm in size. Chitosan (CH) is a biocompatible, and biodegradable natural polysaccharide, which can be used for wall material for forming microcapsules due to their unique cationic property [7]. Functional amino groups on the CH chain are positively charged in acidic solution (pKa = 6.5) [8], thus, their interacting with opposite charged crosslinking agent through ionic gelation can facilitate the formation microcapsules with better mechanical property and protective properties against surrounding condition. This microencapsulation method is the most easy-handled and the produced capsules are highly biocompatible for many applications due to less use of organic solvent, avoids extreme conditions that may cause degradations and/or modifications of bioactive compounds [9]. Due to the pH dependent property for ionization of functional groups [10], lower pHs (less than 3) are encouraged for use in chitosan based microencapsulation. Except for pH, the amount of crosslinking agents [11] and the titration direction of chitosan to crosslinking agent or vice versa may also influence the formation of microcapsules and the encapsulation efficiency [12].

The usage of different anionic crosslinking agent should be taken into consideration for the formation of microcapsules with various properties. The most common anionic crosslinking

agent used for CH based microencapsulation is tripolyphosphate (TPP) salt. This inorganic counterion possess phosphoric ions at pH<3 and can form the inter- or intra-crosslinking with positively charged amino groups in chitosan dissolved in acidic solution. CH-TPP microencapsulation has been widely applied for protecting vitamin C [13], tea polyphenols [14], and delivering tacrine [15], but limited previous study was reported for encapsulating ACN.

Another polymer material, cellulose nanocrystal (CNC) is a rod-like nanoparticle with high crystalline property and negatively charged sulfate (SO_4^{2-}) groups on the surface, they are hydrolyzed from cellulose fibers by using sulfuric acid. Microcapsules formed between CH and CNC have exhibited well-behaved mechanical properties as a vehicle for drugs [12], but no study has been conducted for food ingredient like ACN. In this study, it was hypothesized that ACN loaded CH-CNC microcapsules can stabilize anthocyanins by forming more rigid and stable microcapsules in media than that of CH-TPP based microcapsules by considering their promising mechanical property as drug carriers reported in the former study. In addition, CNC and TPP as anionic crosslinking agents can interact with cationic flavylium form of anthocyanins in low pH, thus entrapping ACN into CH wall matrix.

This study investigated the usage of two different anionic crosslinking agents (CNC and TPP) for forming chitosan based microcapsules to entrap BB ACN through ionic gelation (**Fig. 4.1**). Microencapsulation processes were evaluated through 3 steps. Firstly, the effect of the titration direction on the yield of microcapsules (YOM), total monomeric anthocyanin (TMA) recovery, and other particle characteristics was studied for each type of crosslinking agent. Secondly, YOM and TMA recovery of microcapsules and total phenolic content (TPC), and DPPH radical scavenging activity remained in supernatant were investigated for

identifying the optimal mass ratio between CH and TPP or CNC by modifying different concentrations of anionic crosslinking agent by titrating ACN with CH into CNC or TPP bath. bath. The final part emphasized on evaluating the encapsulation efficiency and ACN distribution in the microcapsules (attached on the surfaces, bound with matrix, or free in the cores) with increasing amount of loaded BB ACN for the optimal formulation of CH-CNC and CH-TPP microcapsules from last study. It is anticipated that this study will provide new insights on the use of CNC as a new potential anionic crosslinking agent for forming more rigid and stable CH based microcapsules for carrying BB ACN.

4.2 Materials and methods

4.2.1 Materials

Sodium tripolyphosphate (TPP) (nominal purity = 98%) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Chitosan was obtained from Primex ehf (Siglufjordur, Iceland) with degree of deacetylation (DDA) of 97.5% and Mw of 300 kDa. Cellulose nanocrystals (CNC) slurry (11.5%, w/v) was provided by the Forest Products Laboratory (FPL), United States Department of Agriculture (USDA). Chitosan, TPP, and CNC were all adjusted to pH 2.6 by 0.1N HCl solution to ensure their ionization and to minimize the structural modification and/or degradation of ACN during encapsulation process.

Folin-Ciocalteu reagent and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), acetic acid and L-ascorbic acid from Avantor Performance Materials (Center Valley, PA, U.S.A.), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (95%) from Alfa Aesar (Ward Hill, MA, U.S.A.). Hydrochloric acid, sodium acetate, potassium chloride and sodium carbonate were all obtained from EMD Chemicals (Gibbstown, NJ, U.S.A.)

4.2.2 Microencapsulation

BB extracts were prepared by using the optimal ultrasound-assisted extraction procedures from our previous study[16]. The extracts were diluted at 1:5 (v/v) with pH 1.0 potassium chloride (KCl) buffer to retain the most stable cationic flavylium form of anthocyanins. Chitosan (0.1% w/w) was dissolved in 1.0% (v/v) acetic acid [17].

The microencapsulation based on the ionic gelation method was applied by following the procedures from Domaratzki and Ghanem [18] with slight modification. Briefly, BB extract (1 mL) was added into 20 mL of 0.1% (w/w) chitosan solution (pH 2.6), the mixture was homogenized using vortex for 1 min. The mixture was then dropwise titrated into TPP or CNC bath (2.0%, pH 2.6) with fierce agitation. After 3 min stirring, the suspensions formed after titration were centrifuged at 10,000 g and 4 ℃ (International Equipment Co., Chattanooga, TN, U.S.A.) for 10 min. Both microcapsules and supernatants were collected for further analysis.

Different influencing factor including the titration direction (BB extract carried by CH (carrier) titrated into TPP or CNC (interactive agent) or *vice versa*, concentration of anionic crosslinking agents (0.025-2.0% of TPP or CNC), and loaded amount of BB ACN (0.40-26.06 mg cyanidin-3-glucoside (Cy-3-Glu) equivalent mg/mL) were all investigated continuously for the addition of each type of anionic crosslinking agent.

4.2.3 Characteristics of microcapsule and supernatant

4.2.3.1 Yield of microcapsules (YOM)

A 50 mL of empty centrifuge tube (VWR International Inc., Bridgeport, NJ, U.S.A.) was weighed at the beginning (m_0), after encapsulation titration process, the produced suspension was transferred to the tube. After centrifugation at 10,000 g (4 ^{o}C) for 10 min, the supernatant was carefully removed and collected for further analysis. While the

microcapsules produced were left at the bottom of tube, the weight of tube together with the microcapsules was recorded (m). YOM obtained from encapsulation process was calculated by by the difference between the two weights (1):

$$YOM\ (g) = m - m_0 \qquad (1)$$

4.2.3.2 Particle size distribution

Particle size and polydispersity index (PDI) of microcapsules in dispersion system were analyzed using the ZetaPALS instrument (Brookhaven Instr. Co., Holtsville, NY). Microcapsules were dispersed in deionized water (1:12.5) and filtered by 0.20 μm filter paper to avoid the influence from microcapsule clusters or air dusts and also guaranteed 300-800 count per second (cps) of each data reading. Prepared microcapsule sample was added into a four-way optical polystyrene cuvette with cap, and the dynamic light scattering was applied. Each microcapsule suspension sample was measured in triplicates with 30 readings, and the mean values of particle size and PDI were generated in 10 min.

4.2.3.3 Total monomeric anthocyanins (TMA) and TMA recovery (%)

TMA was determined by the pH differential method [19] for supernatant / aqueous samples for anthocyanin distribution in the microcapsules study. Collected supernatants/ aqueous samples were appropriately diluted with 3 times of 0.025 M KCl buffer (pH=1.0) and 0.4 M sodium acetate buffer (pH=4.5), respectively. The mixture was vortex and then kept at room temperature for 15 min. The absorbance difference between pH 1.0 and pH 4.5 was determined at both 520 nm and 700 nm using a Shimadzu UV160U spectrometer (Shimadzu Co., Kyoto, Japan). The amount of TMA was expressed as cyanidin 3-glucoside equivalent (Cy-3-Glu) mg/mL since Cy-3-Glu is the most abundant ACN species in fruit materials.

TMA recovery (%) is a parameter that presents the encapsulation efficiency of ACN in the microcapsules, and counts the as TMA percentage for deduction of leached ACN in the supernatant from the original loaded amount of BB ACN to the original loaded amount of BB ACN. Which could be calculated as (Equ. 2):

TMA recovery in microcapsules (%) =

$$\frac{\text{Mass of original TMA (mg in 1 mL BB extracts)} - \text{Mass of remaining TMA in supernatant (mg)}}{\text{Mass of original TMA (mg in 1 mL BB extracts)}} \times 100 \quad (2)$$

4.2.3.4 Analysis of total phenolic content (TPC)

TPC was measured using the Folin-Ciocalteau (FC) assay [20] for supernatant collected after microencapsulation process to reflect the remained amount of TPC in the microcapsules. Briefly, 0.5 mL of collected supernatant after encapsulation and gallic acid (at 0.1, 0.3, 0.5, and 0.7 mg/mL for the standard curve) was mixed with 0.5 mL of FC reagent and 7.5 mL of DI water, respectively. A 0.5 mL of distilled water was used as a control. The mixtures were vortexed at room temperature for 20 min, and then transferred into a 40 ℃ water bath for 20 min with addition of 3 mL of 20% sodium carbonate (w/v) and vortexed. The mixtures were immediately transferred into an ice bath for 3 min for cooling down to room temperature. The absorbance was measured at 765 nm using a Shimadzu UV160U spectrometer (Shimadzu Co., Kyoto, Japan). Results were expressed as gallic acid equivalent (GAE) mg/mL.

4.2.3.5 DPPH radical scavenging capacity (DPPH)

The 2,2-diphenyl-1-picryhydrazyl (DPPH) assay [21] was used to measure the free radical scavenging capacity of polyphenols in the collected supernatants to reflect the DPPH of remained polyphenols in the microcapsules. Briefly, 1 mL of supernatant sample and ascorbic acid at 0.005, 0.01, 0.05, and 0.1 mg/mL (for the standard curve) were mixed with 3

mL of DPPH agent (9 mg DPPH in 100 mL methanol), respectively. The mixture was vortexed at room temperature and kept in the dark for 20 min. The absorbance was then read at 517 nm using the UV/Vis spectrometer (Shimadzu Co., Kyoto, Japan), and the results were reported as ascorbic acid equivalent (AAE) mg/mL.

4.2.3.6 ACN distribution in the microcapsules

During the encapsulation process, BB ACN could bind with any segment of microcapsules including weakly attached on the surfaces of capsules, available freely in the cores, and the rest of ACN bound within the matrix of wall materials and could not be released freely after acid hydrolysis. For monitoring the ACN distribution on these three segments, TMA was determined for aqueous samples from three continuous steps of the treatments on the microcapsules (**Fig. 4.2**). Firstly, microcapsules collected after centrifugation for suspensions formed from encapsulation were washed by 30 mL of water twice, centrifuged, and the aqueous samples was collected for TMA measurements determining ACN attached on the microcapsule surfaces; For releasing the ACN in cores of microcapsules, the same microcapsules were continuously immersed in 30 mL of 0.1 N HCl buffer (pH 1.5) for 24 h, after centrifugation, collected aqueous solution was used to determine TMA [22]. Thirdly, the amount of ACN bound with the microcapsule matrix was calculated by subtracting TMA amount in washed water and HCl aqueous solution together from the amount of TMA remained for the freshly produced microcapsules (equals to the original loading TMA amount minus TMA of supernatant collected after encapsulation). ACN distribution (%) in the microcapsules was then calculated as the percentage of TMA existed in the cores, attached on the surfaces, or bound in the matrix of the microcapsules to the initial amount of loaded TMA.

4.2.3.7 Microscopic image of microcapsules

Freshly made microcapsules (1 g) were dispersed in 100 mL of pH 4.5 buffer, then sonicated and degassed in a Branson B-220H Ultrasound water bath (Branson Ultrasonics Co., Danbury, CT, U.S.A.) for 10 min. After that, one drop of suspension was placed on a glass slide, and the slide was observed under 40 and 100 times of magnification using a Nikon Eclipse 50i microscope (Nikon Corporation, Japan) fitted with an Infinity1-3C camera (Lumenera Co., Atlanta, GA, U.S.A.).

4.2.4 Experimental design and statistical analysis

A completely randomized design was applied for the three-part studies of the experiments, including titration directions between chitosan and crosslinking agents (CNC or TPP), concentrations of anionic crosslinking agents (0.25%-2.5%), and the amount of loaded BB ACN (0.41-26.06 mg cy-3-glu mg /mL), respectively. All measurements were done in triplicatea. Data were analyzed for statistical significance via least significant difference (LSD) *post hoc* test using SAS v9.2 (The SAS Institute, Cary, NC, U.S.A.). Results were considered to be significantly different at $P < 0.05$.

4.3 Results and discussions

4.3.1 Effect of titration direction

The effects of titration direction on the total volume and pH of supernatant and YOM, TMA recovery, and particle characteristics of microcapsules were investigated for two different crosslinking agents (CNC and TPP). Microcapsules were generated from ionic gelation between the cationic chitosan chain and multivalent anionic groups of CNC or TPP. In respect to the counterions used for chitosan based ionic gelation, it can be divided into three groups: high molecular weight counterions (e.g. alginate and κ-carragenan), low molecular weight ions (e.g. lauryl sulphate and cetylstearyl sulphate) and hydrophobic

counterions(e.g. TPP) [23]. Based on the goal to have more sufficient association among crosslinking agents, compounds with smaller molecular weight was used. CNC and TPP classified from two groups were used in the study and anticipated different characteristic property of microcapsules and encapsulation efficiency for anthocyanin.

Comparing two crosslinking agents (**Table 4.1**), BB-CH-CNC suspensions showed significantly (P<0.05) lower total volume and pH than BB-CH-TPP suspension did. This phenomenon could be due to the crystal structure of CNC agent, possibly less interacting happened with hydrogen ion in acidic media than TPP, thus resulting in low pH [24]. In addition, BB-CH-CNC microcapsules produced significantly higher YOM (6.87- 8.13 g) with up to 94% TMA recovery, in comparison with BB-CH-TPP microcapsules with YOM of 0.27-0.67 g and TMA recovery of 32-42%. The higher YOM in the use of CNC was also observed by Shu and Zhu [25] that the microcapsules formed from sulphate crosslinked CH-gelatin were smoother and larger with hydration, whereas large gaps indicating weaker complex were observed in CH-TPP microcapsules in the same study.

Regarding the effect of titration direction for each type of crosslinking agent, CNC as a carrier titrated into CH showed significantly (P<0.05) higher YOM than that from the opposite direction, which might be due to repulsive forces among CH chains with more water molecules [26]. In TPP bath, when CH as a carrier of BB extracts and titrated into TPP, the suspensions showed significantly (P<0.05) higher pH compared to that from the opposite direction. This could be explained as TPP could possibly interact with hydrogen ions from acetic acid in chitosan solutions, thus resulting in higher pH.

In general, BB-CH-TPP microcapsules were significantly larger (up to 34 μm), compared to the BB-CH-CNC microcapsules (from 65 to 283 nm). BB-CH-TPP microcapsules were much

larger than those microcapsules produced in the previous drug delivery study [15], which could be due to the aggregation of formed particles with less charged compounds, compared to BB-CNC-CH microcapsules as better dispersed colloids. BB-CNC-CH microcapsules were similar to and even smaller than the CNC-CH polyelectrolyte complex from the study of Wang & Roman [12], showing 265-969 nm at different mass ratios of CNC to CH. In addition, CNC as a carrier of BB extracts and titrated into CH could result in low PDI of 0.22 (< 0.3), considering as ideal and narrow size distribution of formed microcapsules [27]. This finding can be provided as a suggestion for modifying the microcapsules on the purpose to achieve better mechanical properties.

However, by considering our aim to achieve highest TMA recovery of produced microcapsules, as well as the results proved that no significant difference of TMA recovery showed for two titration directions in same formulation. We applied the encapsulation process with using CH as a carrier of BB ACN extracts and titrating into CNC or TPP bath for further studies. The effects of concentration of CNC or TPP and the amount of loaded BB ACN on YOM, TMA recovery, TPC, and DPPH of microcapsules or supernatants were investigated as follows.

4.3.2 Effect of different concentrations of anionic crosslinking agent

As stated above, for both TPP and CNC, CH as a carrier of BB ACN and titrated into anionic crosslinking agent was selected, and YOM and TMA recovery of formed microcapsules and TPC and DPPH of supernatants were further investigated for the effect on different concentrations of anionic crosslinking agents added (**Fig. 4.3**). Since our goal for this study was to obtain high YOM, TMA recovery of the microcapsules, and less amount of TPC and DPPH remained in the supernatants. And the lower TPC and DPPH values in the

supernatant indicates higher efficient encapsulation process with more BB ACN and polyphenols entrapped in the microcapsules. While using CNC as the crosslinking agent, the increasing concentration of CNC induced higher YOM from 1.28 to 8.03 g and TMA recovery from 40 to 96%, significantly increase starting from CNC concentration of 1.0% (w/v) (**Fig. 4.3a**), and showed further significant (P<0.05) increase of YOM up to ~12.42 g at the concentration of 2% (w/v). It was observed that TPC remained in the supernatant was higher when lower concentration of CNC was used (**Fig. 4.3b**), which might be related to that there was no available CNC for binding free ACN due to the excess amount of CH in the aqueous media. Then, a sharp reduction of TPC was observed at 1.0% CNC (**Fig. 4.3b**), indicating more TPC started entrapping inside the microcapsules from that concentration. DPPH result also showed similar trend with TPC. Hence, CNC at 1.0% was chosen for making BB-CH-CNC microcapsules with higher encapsulation efficiency and less use of the crosslinking agent. For BB-CH-TPP microcapsules, 1% of TPP was also selected as the optimal concentration as it showed the highest YOM (~1 g) (**Fig. 4.3c**). Hence, the mass ratio of CH (0.1% concentration) to both anionic crosslinking agents was both determined as 1:10. This mass ratio was much higher than other previous encapsulation study, in which CH: TPP = 4:1 for microencapsulation was found as the best formulation for the delivery of bovine serum albumin (BSA) [28] and CNC: CH = 4:1 or 8:1 also reported forming a capsules for delivering another drug with good mechanical property [12]. This difference could be because the cationic ACN at low pH (pH <3) can interact with anionic crosslinking agents as well in the process and be entrapped into wall material matrix.

4.3.3 Effect of loaded amount of blueberry (BB) extracts

Freshly prepared BB ACN extracts were diluted into concentrations at the range of 0.40-26.06 cy-3-glu mg/mL by dilution. **Fig. 4.4** shows the effect of increasing amount of loaded BB

ACN on YOM and TMA recovery in the microcapsules and TPC and DPPH remained in the supernatants after encapsulation process. For BB-CH-CNC suspensions, the amount of BB ACN extracts had no significant effect on YOM and TMA recovery of microcapsules and remained TPC in supernatant **(Fig. 4.4a)**, whereas DPPH in supernatant was significantly increased when loading 26.06 mg cy-3-glu mg/mL ACN (**Fig. 4.4b**). This odd observation could be caused by the color interference from condensed BB ACN, thus impacting the absorbance at 517 nm. Using CNC as anionic crosslinking agent for CH based microencapsulation illustrated high encapsulation efficiency (68-93%) for loading BB ACN in the range we targeted in this study.

For BB-CH-TPP suspensions, no significant difference in YOM of microcapsules was observed among different amounts of loaded BB ACN. However, TMA recovery was found none significant difference when lower amount of loaded BB ACN was presented. However, significant decrease of TMA recovery (from 40% to 20%) was observed at the concentration of 26.06 cy-3-glu mg/mL **(Fig. 4.4c)**. Consistently, an increase of TPC and DPPH in the supernatants were found when the concentration of BB ACN reach to 26.06 cy-3-glu mg/mL. (**Fig. 4.4d**). This findings indicated the worse encapsulation efficiency and less binding happened when the extra ACN amount was loaded. The maximal loading amount of BB ACN should be no more than 14.23 cy-3-glu mg/mL for TPP-CH-BB microcapsules.

4.3.4 ACN distribution in the microcapsules

ACN distributions in the microcapsules, including the segment of ACN freely entrapped in the cores, bound with matrix, and attached on the surfaces, were investigated for understanding the formation of microcapsules with interactions between wall materials and ACN depending on type of anionic crosslinking agent applied and loaded amount of BB ACN. This result would

help to explain the stability of the ACN in microcapsules, as encapsulated ACN on surface and in matrix can be easily degraded and/or harder to release in food application with sufficient hydrolysis. Different studies were operated on specific purpose. Unlike the application of microcapsules for drug delivery, where the researcher determined the well-performed microcapsules with minimal loading amount of saffron extract with high encapsulation efficiency for slow releasing rate after oral consumption[29]. Our study are more focus on how many free original anthocyanins available in core areas.

As shown in **Fig. 4.3**, ACN distribution depended on both the type of crosslinking agents and the amount of loaded BB ACN. For BB-CH-CNC microcapsules, as reported above, 68-93% TMA recovery of microcapsules could be obtained from a wide range of loaded BB ACN, but their ACN distributions varied. At the BB ACN loading range of 0.41-0.84 cy-3-glu mg/mL, ACN was fully bound with CNC, whereas ACN started accumulated on surface of microcapsules when reached to 2.38 cy-3-glu mg/mL loading BB ACN **(Fig. 4.5A)**. Within ACN range of 6.09-26.06 cy-3-glu mg/mL with more loading, there was a trend that of growing ACN (15 to 34%) attached on the surface. Moreover, less ACN was found bound in the matrix with more ACN presented in the cores. These results indicated the formulation of BB-CH-CNC could induce a core-shell type of microcapsules as majority of freely existed ACN in the core could be achieved as long as certain amount of ACN was loaded.

For BB-CH-TPP suspensions, TMA recovery reached up to 40% (**Fig. 4.4**), but only less than 5% of ACN were found in the cores or attached on the surfaces (**Fig. 4.5B**). This might be due to the high reactivity and flexibility of TPP, thus their interaction with ACN facilitate the more crosslinked ACN entrapped in CH based wall matrix. Considering our preference for remaining more freely available colorant inside the cores of microcapsules. We concluded that

the use of TPP as anionic crosslinking agent in encapsulation is lack of encapsulation efficiency as more anthocyanin directly bound with matrix compared to the application of CNC.

4.3.5 Microscopic images of microencapsulates

As shown in **Fig. 4.6**, the microscopic images of the microcapsules indicated their morphology characteristics are totally different with each other for the formulation of BB-CH-CNC and BB-CH-TPP. The spherical microcapsules were observed in BB-CH-CNC microcapsules with clear entrapment of red colors. The irregular morphology of BB-CH-TPP microcapsules with holes among crosslinked joints in the cluster were present in the image, this finding explained that the limited encapsulation efficiency for this formulation is related to their porous structure.

Conclusions

This study demonstrated that the type of applied anionic crosslinking agents and titration directions had significant effects on the yield of microcapsules, encapsulation efficiency, and particle characteristics. TMA recovery for both titration direction showed no significant difference within the same crosslinking agents. Cellulose nanocrystal as a crosslinking agent for forming chitosan based microcapsules proved providing high encapsulation efficiency (up to 94% of TMA recovery). The light microscopic images clearly showed that anthocyanins were able to be entrapped in the formed microcapsules. The concentration of CNC or TPP at 1.0% (w/v) significantly improved YOM and TMA recovery with the least TPC and DPPH remained in the supernatant. The loading amount of blueberry anthocyanins had no significant influence on TMA recovery, but impacted the anthocyanins distribution in the microcapsules. This study successfully demonstrated the use of CNC as an anionic

crosslinking agent for chitosan based microencapsulation could form more small, rigid and stable microsphere for loading blueberry anthocyanins.

For future studies, developed BB-CH-CNC microcapsules should be investigated in their stability during the storage under various surrounding conditions (temperature, pH, and light). Also, other anthocyanin-rich plant extracts with various loading form (powder or extract) could be investigated in future for optimizing their encapsulation efficiency. In additions, the applications of the developed anthocyanin microcapsules in various food products, such as condiments, yoghurt, drinks, or baked goods, should be also conducted to reveal more practical guidelines on using the encapsulated anthocyanins.

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Table 4.1 Effects of different anionic crosslinking agents (cellulose nanocrystal (CNC) and sodium tripolyphosphate (TPP)) and titration directions on blueberry extract (BB)-loaded chitosan (CH)-based encapsulation suspension and their microcapsule properties

Crosslinking agents		Supernatant of encapsulation suspension	Microcapsules				
Carrier*	Interactive agent	Total volume (mL)	pH	Yield (g)	TMA* recovery (%)	Effective diameter (nm)	Polydispersity Index (PDI)
A) BB-CH-CNC							
CH	CNC	30.67^{b+}	2.23^{a}	6.87^{b}	94.02^{a}	64.80^{b}	1.05^{a}
CNC	CH	29.67^{b}	2.19^{a}	8.13^{a}	85.42^{a}	282.97^{a}	0.22^{b}
B) BB-CH-TPP							
CH	TPP	38.67^{A}	2.53^{A}	0.27^{A}	32.54^{A}	33887.00^{A}	0.66^{A}
TPP	CH	38.17^{A}	2.47^{B}	0.67^{A}	42.33^{A}	10320.43^{B}	0.40^{B}

Encapsulation suspension was prepared with 1 mL of BB extracts (with pH 1.0 buffer diluted), 20 mL CH solution (0.1% in 1% acetic acid, pH 2.6), and 20mL of TPP or CNC solution (2.0 %, pH 2.6).

Both supernatant and microcapsules were collected for analysis after centrifugation.

Titration direction: Carrier with BB extract was dropwise transferred into the flask containing interactive agent.

*TMA recovery (%) $=\frac{\textbf{TMA in 1 mL of BB} - \textbf{Leached TMA in encapsulation supenatant}}{\textbf{TMA in 1 mL BB}} \times 100$ (%), in which total monomeric anthocyanin (TMA, mg Cyanidin-3-glucoside equivalent/mL) was calculated for the collected supernatant.

+ Means preceded by the same small letter in the same column were not significantly different with each other (P>0.05) (n=3).

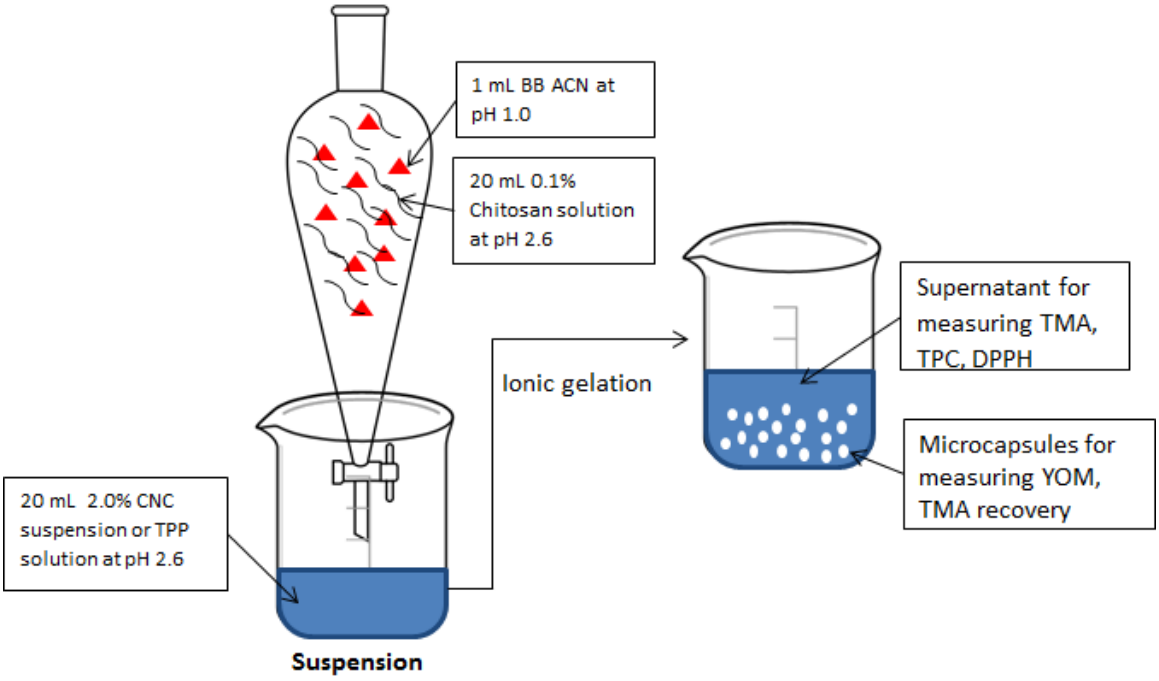


Fig. 4.1 The diagram for encapsulation process through ionic gelation

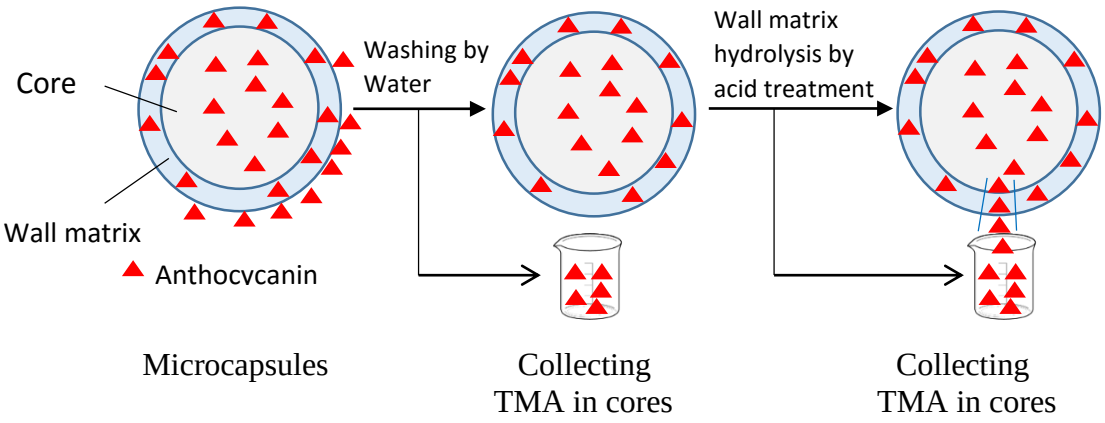
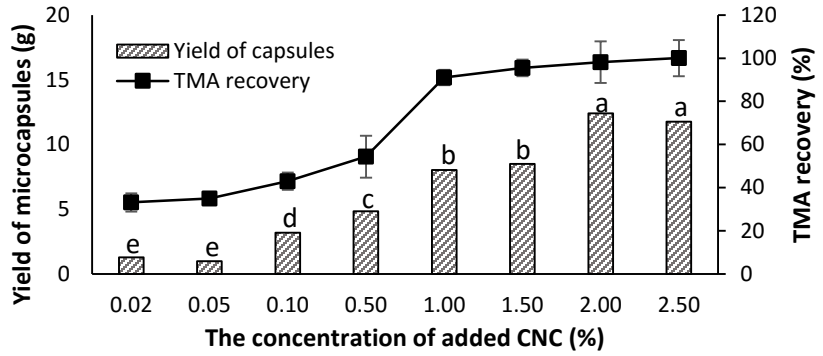


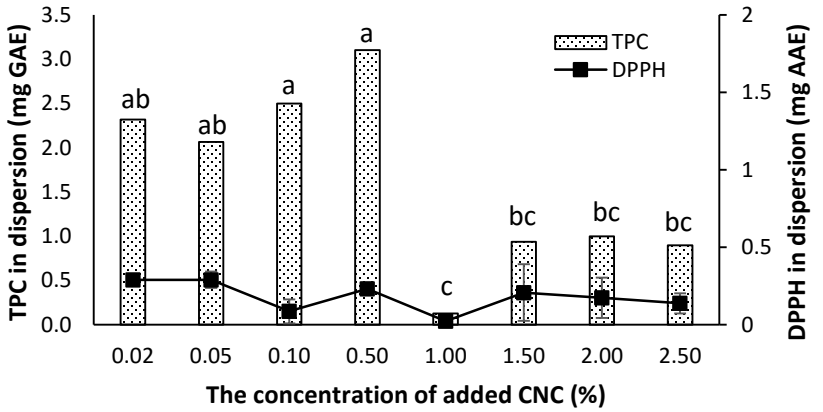
Fig. 4.2 The schematic diagram for anthocyanin distribution study

BB-CH-CNC formulation

(a)

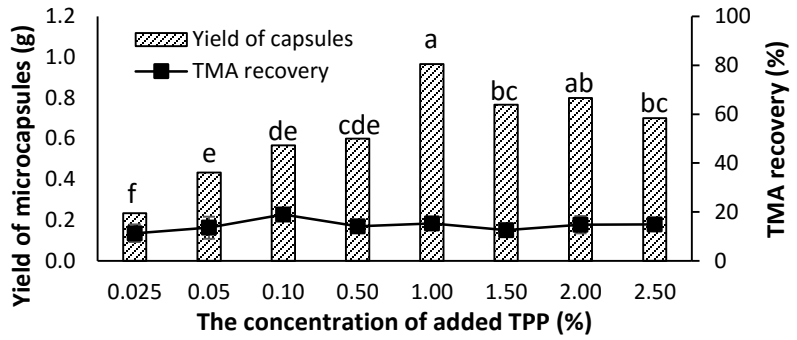


(b)

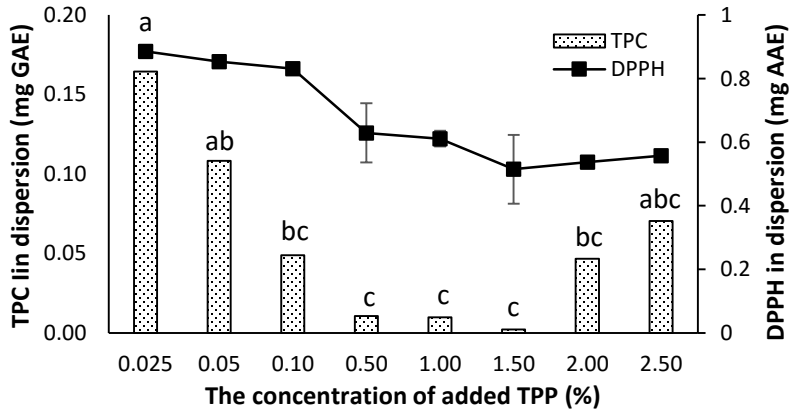


BB-CH-TPP formulation

(c)



(d)



(Continued the Figures in last page)

Fig. 4.3 Effects of types of anionic crosslinking agents (cellulose nanocrystal (CNC) or sodium tripolyphosphate (TPP)) and their concentrations on the yield of microcapsules, total monomeric anthocyanin (TMA) recovery (%), total phenolic contents (TPC), and antioxidant (DPPH) activity in blueberry extract (BB) loaded chitosan (CH)-based microencapsulation

BB extracts for CNC added group contain TPC of 0.04 ± 0.01 mg GAE and DPPH of 0.05 ± 0.00 mg AAE; The BB for TPP added group contain TPC of 0.15 mg ± 0.00 GAE and DPPH of 0.05 ± 0.00 mg AAE.

Encapsulation suspension was formed as follows: 1 mL BB extracts (with pH 1.0 buffer diluted) added to 20mL CH solution (0.1% in 1% acetic acid, pH 2.6) were dropwise transferred to the flask containing 20 mL TPP (0.025%, 0.05%, 0.1%, 0.5%, 1.0%, 1.5% and 2.0%, pH 2.6) or CNC (0.02 %, 0.05%, 0.1%, 0.5%, 1.0%, 1.5% and 2.0%, pH 2.6).

Yield of capsules indicated the total weight of capsules obtained from the microencapsulation process.

TMA determined by pH differential measurement of total monomeric anthocyanin (mg Cyanidin-3-glucoside equivalent/mL).

$$\text{TMA recovery (\%)} = \frac{\textbf{TMA in 1 mL of BB} - \textbf{Remained TMA in suspension}}{\textbf{TMA in 1 mL BB}} \textbf{ x 100}$$

Total phenolic content (TPC, gallic acid equivalent (GAE)) and DPPH radical scavenging capacity (ascorbic acid equivalent (AAE)) were measured for the remaining supernatant.

The same letter above the bar chart in each measurement indicated no significant difference among levels (P>0.05).

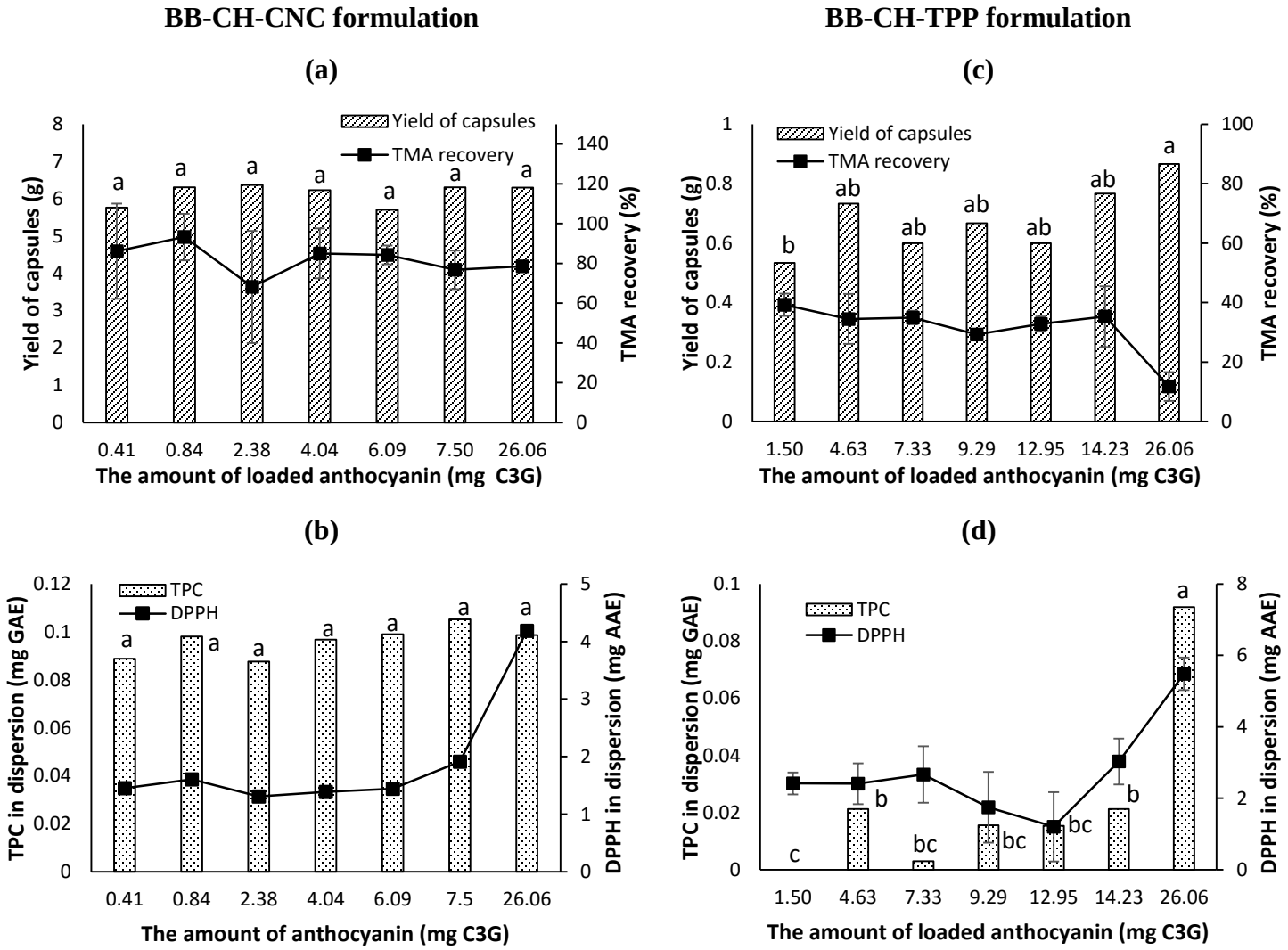


Fig. 4.4 Effects of types of anionic crosslinking agents (cellulose nanocrystal (CNC) or sodium tripolyphosphate (TPP)) and their blueberry extracts loading amount on yield of capsules, total monomeric anthocyanin (TMA) recovery (%), total phenolic contents (TPC), and antioxidant (DPPH) activity in blueberry extract (BB) loaded chitosan (CH)-based encapsulation

Fig. 4.4 (Continued)

*The optimal conditions for chitosan based microencapsulation are 1 mL BB extracts + 20 mL CH solution (0.1% in 1% acetic acid, pH 2.6) + 20 mL TPP %, 1.0%, pH 2.6) or CNC solution (1.0%, pH 2.6) decided from Fig.3

* Yield indicated the total wet weight of capsules obtained from the microencapsulation process.

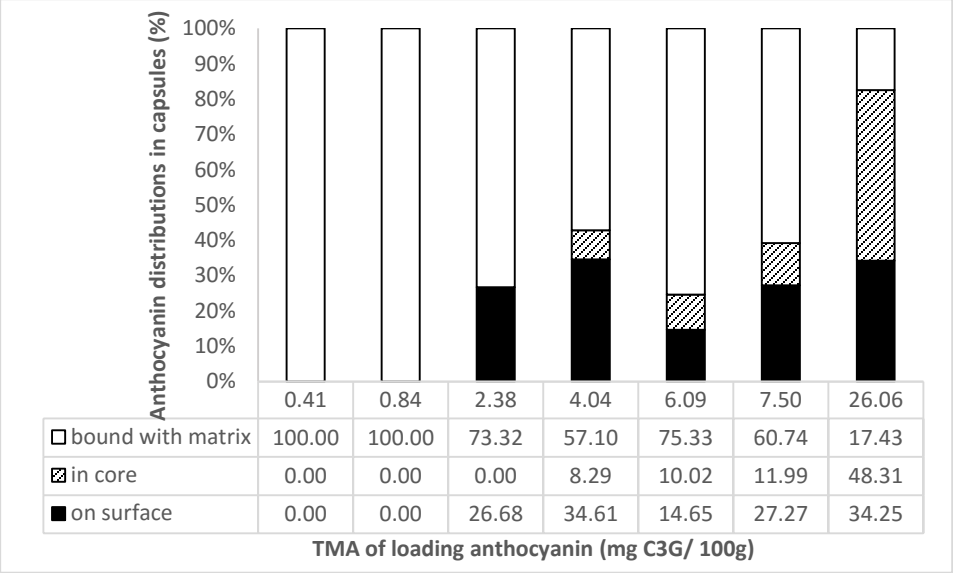
* Total monomeric anthocyanin (TMA) determined by pH differential measurements for encapsulation solution after the capsules formed, mg Cyanidin-3-glucoside equivalent/g (mg C3G/100g).

$$\text{TMA recovery (\%)} = \frac{\textbf{mass of original TMA (mg in 1 mL BB extracts)} - \textbf{mass of remaining TMA in encapsulation solution (mg)}}{\textbf{mass of original TMA (mg in 1 mL BB extracts)}} \times \mathbf{100}$$

* Total phenolic content (TPC) and DPPH radical scavenging capacity were measured for both the original blueberry extracts and the remaining encapsulation solution. The units for them are mg Gallic acid equivalent (GAE) and mg Ascorbic acid equivalent (AAE) by times the exact volume of extracts/ solution.

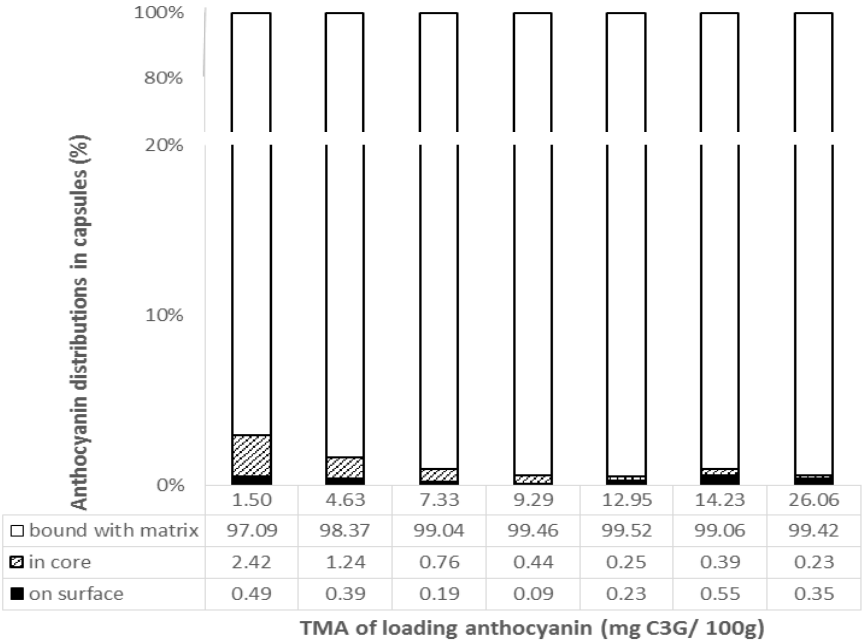
+ The same lowercase or uppercase letter above histograms in each measurement indicated no significant difference detected by LSD test among levels for the same factor (P>0.05).

A) BB-CH-CNC



	0.41	0.84	2.38	4.04	6.09	7.50	26.06
bound with matrix	100.00	100.00	73.32	57.10	75.33	60.74	17.43
in core	0.00	0.00	0.00	8.29	10.02	11.99	48.31
on surface	0.00	0.00	26.68	34.61	14.65	27.27	34.25

B) BB-CH-TPP



	1.50	4.63	7.33	9.29	12.95	14.23	26.06
bound with matrix	97.09	98.37	99.04	99.46	99.52	99.06	99.42
in core	2.42	1.24	0.76	0.44	0.25	0.39	0.23
on surface	0.49	0.39	0.19	0.09	0.23	0.55	0.35

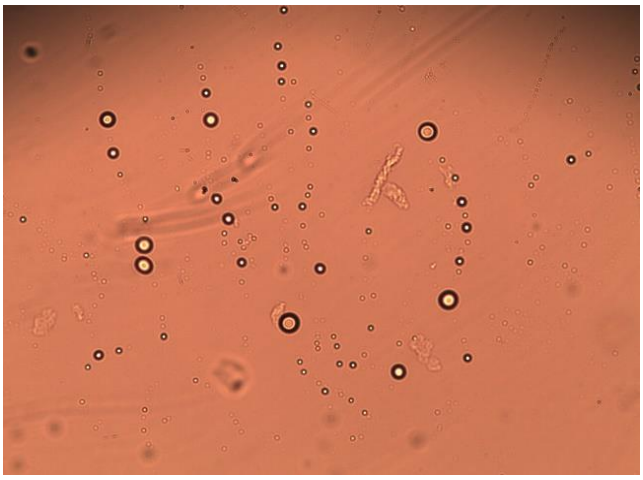
Fig. 4.5 The distribution of anthocyanins in whole microcapsules (on surface, in the matrix, and inside the core) with increasing blueberry anthocyanin loading concentration in the optimal microencapsulation conditions using cellulose nanocrystal (CNC) or sodium tripolyphosphate (TPP) as a anionic gelling agent (N=3).

(Fig. 4.5 Continued)

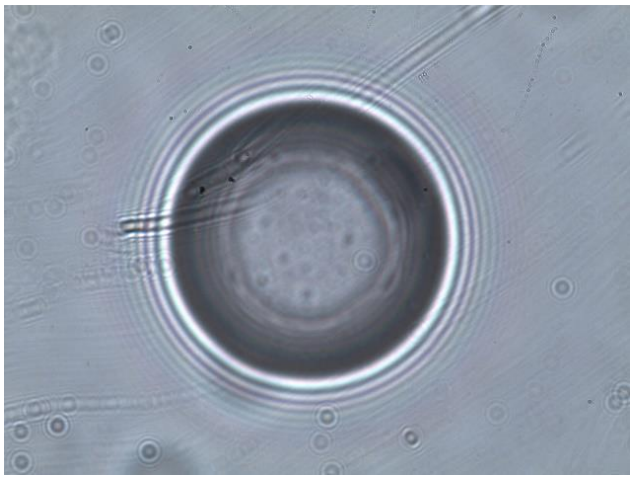
*The optimal conditions for chitosan based microencapsulation are 1 mL BB extracts + 20mL CH solution (0.1% in 1% acetic acid, pH 2.6) + 20mL TPP %, 1.0%, pH 2.6) or CNC solution (1.0%, pH 2.6).

**All measurements were determined by TMA. The anthocyanin on surface indicted the amount of TMA remaining in the two times washing water (~30mL in total), the anthocyanin remained in core without any binding is decided by the TMA measurements on the washing solution of 0.1N HCl after 20h shaking. Results were showed in the mean value of triplicated measurements and presented as a percentage of original TMA of loading anthocyanin

A) BB-CH-CNC

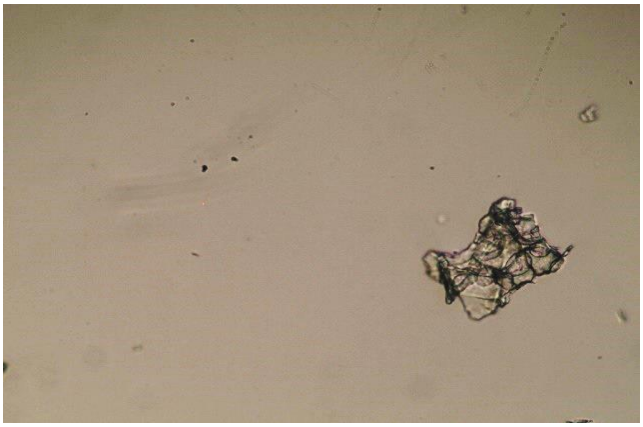


Magnification 40x

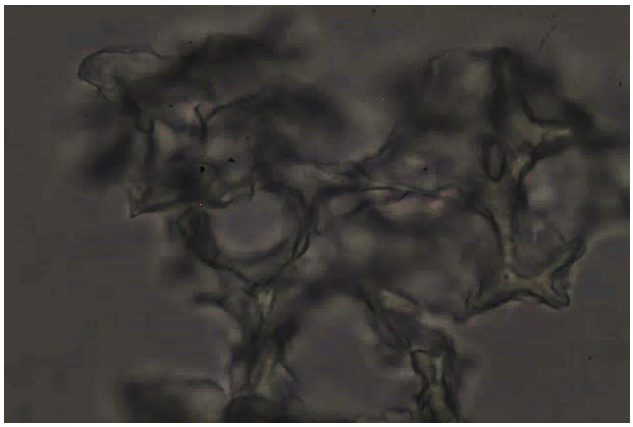


Magnification 100x

B) BB-CH-TPP



Magnification 10x



Magnification 40x

Fig. 4.6 Microscopic image for microcapsules morphology

*All microscopic image was taken by the microscope with the optimal encapsulation formulation. Since CNC is pH dependent for ionization, 1 g of wet capsule sample was diluted by 10 volume of pH 4.5 buffer. For TPP-Chitosan group, only water was used to dilute capsule samples. Ultrasound was used to disperse the bubbles in the solution.

CHAPTER 5

General Conclusions

This study identified the optimal extraction conditions and microencapsulation formulations for extracting and stabilizing anthocyanins and polyphenols from fruit materials, respectively. The results provide comprehensive information on the procedures and methods to enhance extraction and improve the stability of natural anthocyanin pigments from different fruit. materials.

While using conventional solvent extraction (CE) and ultrasound-assisted extraction (UE) methods for blueberries, cherries, and red pear peels that have different anthocyanin compositions, the optimum extraction conditions were identified as: 60% methanol, 50 ℃ for 1 h using CE or 70% methanol, 30 ℃ for 20 min using UE for blueberries; 60% ethanol, 70 ℃ for 1 h using CE or 80% ethanol, 30 ℃ for 20 min using UE for cherries; 60% methanol, 50 ℃ for 1 h using CE or 60% ethanol, 30 ℃ for 60 min using UE for red pear peels. It was found that UE method can enhance the polymerization of anthocyanins for blueberry and red pear peel extracts and increase the total monomeric anthocyanins in cherry extracts. The ultrasound assistance may be more suitable for materials with firm cell wall structure for effective cell disruptions, thus

allowing the use of low concentration of organic solvent and lower temperature for anthocyanin extraction. HPLC analysis also revealed that both extraction methods altered the amount of individual anthocyanin compositions in the extracts, including the structure in the form of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, or malvidin derivatives. It was concluded that different conditions for CE and UE should be implemented for fruit with different anthocyanin compositions for maximizing the recovery of anthocyanins. In the future studies, additional extraction assisting technologies, such as microwave, high hydrostatic pressure, might be evaluated, and other types of anthocyanin rich fruit with different anthocyanin compounds should also be investigated using the developed optimal extraction conditions.

The chitosan (CH)-based microencapsulation study revealed that the type of applied anionic crosslinking agents (tripolyphosphate (TPP) or cellulose nanocrystal (CNC)) influenced the yield of microcapsules (YOM), encapsulation efficiency, and particle characteristics for the produced microcapsules. Titration direction of the blueberry anthocyanins (BB ACN) and carrier agent in form of the microencapsulation suspension did not show significant effect on TMA recovery as long as the same encapsulation formulation was applied. While using CNC as an anionic crosslinking agent, the formed BB-CH-CNC microcapsules possessed high encapsulation efficiency (up to 94% of TMA recovery) than that with TPP as a crosslinking agent. While developing the optimal encapsulation formulation using CH as the carrier for BB ACN, it was found that when the concentration of the anionic crosslinking agent risen up to 1.0% (w/v) in the suspension (the mass ratio of

chitosan : anion=1:10), both BB-CH-CNC and BB-CH-TPP encapsulation formulations showed improved capsule quality with high TMA recovery and and the less binding of TPC and DPPH with microcapsules was found. The light microscopic images for the microcapsules clearly showed that anthocyanins were able to be entrapped in the formed BB-CH-CNC microspheres, while BB-CH-TPP formed a porous and irregular microcapsules. The amount of loaded BB ACN had no significant effect on TMA recovery and YOM as long as the amount of BB ACN loaded was below 26.06 mg Cyanidin-3-glucoside equivalent mg/mL for the formulation using BB-CH-TPP, and increasing loaded BB ACN did impact the distribution of anthocyanins in the microcapsules as BB-CH-CNC formulation could form a core-shell type microcapsules with most of ACN freely existed in the cores (up to 48%). However, BB-CH-TPP formed microcapsules with majority of the encapsulated anthocyanins (over 95%) bound in matrix over the loading range and having difficult for efficient release. This study successfully demonstrated the use of CNC as an anionic crosslinking agent for chitosan based microencapsulation by forming small, rigid and stable microcapsules entrapping BB ACN. In the future, other fruit extracts should also be investigated as different crosslinking reactions may occur depending on anthocyanin species and concentration among fruit. In addition, the effect of different types of anthocyanin samples (juice, extract or dried powders) should be investigated for their influence on the encapsulation efficiency. The applications of these capsules on food application, such as yoghurt, beverage, or baked goods should also be studied to reveal more practical guidelines on using the stabilized anthocyanin pigments.

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