DEVELOPMENT OF AN ORAL DELIVERY SYSTEM TO MODULATE THE RELEASE OF ANTHOCYANINS EXTRACTED FROM HASKAP BERRIES (*LONICERA CAERULEA* L.)

by

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DEDICATION

For their encouragement and support, this thesis is dedicated to:

my parents, Ivo and Jussara

my husband, Luiz

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ABSTRACT

Anthocyanins (ACNs) can be related to positive health benefits and are naturally present in many fruits. Haskap berries (*Lonicera caerulea* L.) have been traditionally associated with therapeutic properties, and as they are particularly rich source of ACNs, have the potential to develop into value-added products that are designed to maximize health benefits. In this thesis, a novel strategy to modulate the release of ACNs is proposed. This approach seeks to increase ACN retention in the stomach by designing a controlled delivery system to modulate the overall release and absorption of ACNs in the body, and reduce degradation due to intestinal pH or metabolism. Firstly, a theoretical physiologically-based, multi-compartmental pharmacokinetic (PBPK) model is developed to describe the fate of ACNs *in vivo*, where the role of the stomach and small intestine is recognized. Then, based on the evidence from recent literature, a strategy using gastroretentive systems as a platform for delivery of ACNs for therapeutic use (with type 2 diabetes as a model of degenerative disease) is proposed.

Two novel gastroretentive systems (floating microspheres and an *in situ* gelling raft) were developed for oral delivery of ACNs. Firstly, parameters for ultrasound-assisted extraction of ACNs from haskap berries were studied using Response Surface Methodology (RSM). For the microspheres, ACN-rich extract was incorporated into non-floating calcium-alginate microparticles formed by extrusion/gelation method and optimized by RSM. Then the procedure was modified to incorporate calcium carbonate for gas generation to produce floating beads, where increasing the carbonate/alginate weight ratio from 0 to 3:4 resulted in different degrees of floatability, larger particles, higher encapsulation efficiency, and lower ACN release. The *in situ* gelling raft system with ACN-rich extract demonstrated suitable gelling and release characteristics. In both cases, the release of ACNs from the system was modulated where diffusion was the dominant mechanism. The raft system demonstrated a more sustained release of ACNs over time than the microspheres, and is recommended over the microspheres, due to simpler processing requirements and ease for scale-up. Thus, the *in situ* gelling system is recommended for further development.

LIST OF ABBREVIATIONS AND SYMBOLS USED

$A_{x\_Lu_i}$	Amount of parent ACN (*i*) in the lumen of a given compartment (*x*)
A_{x_i}	Amount of parent ACN (*i*) in a given compartment (*x*) or in the ingested food (*F*)
AAE	Ascorbic acid equivalents
ABTS	2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid radical
ACN	Anthocyanin
ADME	Absorption, distribution, metabolism, and excretion
AE	Active renal elimination
ALA	α-linolenic acid
ANOVA	Analyses of variance
AUC	Area under the curve (amount *x* time/volume)
BB	Box–Behnken design
BCRP	Breast cancer resistance protein
BE	Biliary excretion
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BW	Body weight
C3G	Cyanidin 3-glucoside equivalents
C3Rut	Cyanidin 3-rutinoside equivalents
C_{B_i}	Concentration of the parent ACN in blood (amount/length^3)
C_{max}	Maximal plasma concentration
C_{x_i}	Concentrations of ACNs in a given compartment (*x*) (amount/length^3)
CFU	Colony forming units
CL_{K_i}	Renal clearance of the parent ACN (volume/time)

CL'_{Li_i}	Intrinsic clearance with respect to the parent ACN concentration in the liver (*Li*) (volume/time)
COMT	catechol-*O*-methyltransferase
COX-2	Cyclooxygenase-2
CV%	Coefficient of variation
D	Overall desirability function
d_i	Desirable value
DAD	Diode array detector
DE	Dextrose equivalent
DHA	Docosahexaenoic acid
DPPH	2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl radical
DW	Dry weight basis
EC_{50}	Effective concentration
ED	Electrochemical detection
EE	Encapsulation efficiency
EHC	enterohepatic circulation
EPA	Eicosapentaenoic acid
f_{B_i}	Fraction of free parent ACN in the blood
f_1	Difference factor
f_2	Similarity factor
FC	Folin–Ciocalteu
FDS	Floating delivery system
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
G6Pase	Glucose-6-phosphatase
GAE	Gallic acid equivalents
GC	Gas chromatography

GI	Gastrointestinal
GLUT4	Glucose transporter 4
GRAS	Generally recognized as safe
GRS	Gastroretentive system
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
I	Small intestine
IC_{50}	Inhibitory concentration
IL	Interleukin
K	Kidneys
k	Kinetic constant
$K_{a_{x_i}}$	Absorption rate constant of the parent ACN in a given compartment (time^{-1})
k_e	Renal excretion rate constant (time^{-1})
K_{m,x_mi}	Michaelis–Menten constant (amount/length^3)
L	Large intestine
LDL	Low-density lipoprotein
Lg	Lungs
LPH	Lactase phlorizin hydrolase
LPS	Lipopolysaccharide
Lu	Organ lumen
M	Mucosa
MMC	Migrating myoelectric complex
MS	Mass spectrometry
n	Release exponent
NADH	Nicotinamide adenine dinucleotide

NF-κB	Nuclear factor-κB
ORAC	Oxygen radical absorbance capacity
P	Percentage contribution
$P_{B:Air}$	Blood:air partition coefficient
P_t	Number of time points
$P_{x:B}$	Partition coefficient of the ACN between the compartment (x) and blood
PB	Plackett–Burman design
PBPK	Physiologically-based pharmacokinetic
PK	Pharmacokinetics
p*K*a	Acid dissociation constant
Q_c	Cardiac output (volume/time)
QE	Quercetin equivalents
Q_v	Alveolar ventilation (volume/time)
Q_x	Blood flow rates to the compartment (x) (volume/time)
r	Pearson's correlation coefficient
R^2	Regression coefficient
ROS	Reactive oxygen species
RP	Rapidly perfused tissues
RSM	Response Surface Methodology
S	Standard error of the regression
SGLT	Sodium-glucose co-transporter
SP	Slowly perfused tissues
St	Stomach
SULT	Sulfotransferases
T2D	Type 2 diabetes
$t_{1/2}$	Half-life (time)

T_g	Glass transition temperature
t_{max}	Time to reach maximum plasma concentration (C_{max})
TAC	Total anthocyanins content
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tertiary-butyl hydroquinone
TE	Trolox equivalent
TEAC	TE antioxidant capacity
TFC	Total flavonoid content
TNF-α	Tumor necrosis factor alpha
TPC	Total phenolic content
TPP	Tripolyphosphate
UAE	Ultrasound-assisted extraction
UDPGDH	Uridine diphosphate-glucose dehydrogenase
UDPGT	Uridine diphosphate-glucuronosyltransferases
USP	U.S. Pharmacopeial Convention
V_i	Volume of distribution of the parent ACN (volume)
V_{max,x_mi}	Apparent maximum velocity or the theoretical maximum metabolic (amount/time)
V_x	Volume of the compartment (x) (% body weight)
ξ_i	Natural variable/factor

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Chapter 1 INTRODUCTION

Research has provided evidence that supports the use of foods as active agents in the prevention or amelioration of nutrition-related diseases and improvement of the quality of life (Beattie, Crozier, & Duthie, 2005; Oyebode, Gordon-Dseagu, Walker, & Mindell, 2014). Recent *in vitro* and *in vivo* studies have shown that the consumption of a variety of berries can be associated with a number of positive health-related effects, including the reduction of cancer (Gordillo et al., 2009), improved hyperglycemia and insulin resistance (Stull, Cash, Johnson, Champagne, & Cefalu, 2010; Vuong et al., 2009), reduction of bone loss (Devareddy et al., 2008), and neurocognitive benefits (Krikorian et al., 2010). However, the incorporation of typical bioactive compounds (e.g. vitamins, lipids, and peptides) in different food matrices is limited by their inherent characteristics, such as taste and incompatibility with the formulation and its ingredients, and subsequent processing that can affect the retention of labile compounds (Augustin & Hemar, 2009).

In this respect, encapsulation is a versatile technology that can address these market needs, contributing to the development of high-value food products (Betoret, Betoret, Vidal, & Fito, 2011). Encapsulation processes are used to entrap one substance (termed core material or active agent) within another (coating, shell or carrier/wall material) (Zuidam & Shimoni, 2010), and there are many different techniques that can be used to achieve this. Encapsulation techniques can contribute to extend the product's shelf-life, protecting the active components against degradation during storage and maintaining their functionality; mask unwanted flavours, smell or taste (Martin & Appel, 2010); and increase the effectiveness of natural functional compounds that normally have a lower potency at equivalent levels when compared to synthetic ingredients (McClements, Decker, Park, & Weiss, 2009).

Anthocyanins (ACNs) are bioactive molecules that can benefit greatly from encapsulation, as their stability is often affected by environmental conditions, such as pH, temperature, oxygen (Rein, 2005), light, heat (Wrolstad, 2004), water activity (Garzón & Wrolstad, 2001), and presence of other compounds (e.g. phenolics, enzymes, metal ions, sugars, ascorbic acid) (Skrede, Wrolstad, Lea, & Enersen, 1992). ACNs are members of

the flavonoid class of secondary metabolites and important water-soluble pigments in plants (Bueno et al., 2012; Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). In addition to their colorant properties, several studies have related the consumption of these compounds to health benefits (Cvorovic et al., 2010; Jennings, Welch, Spector, Macgregor, & Cassidy, 2014; Roy et al., 2002). In this context, haskap berries (*Lonicera caerulea* L.) have gained the attention of the scientific community due to the high levels of ACNs in comparison to other known sources, in addition to their vitamin C content. These fruits are native to northeastern Asia, where they are recognized as the *elixir of life* by Japanese aboriginals, and were recently introduced in the North America market (Bors et al., 2012). Different health-related benefits have been associated with these berries using *in vitro* and *in vivo* studies (Celli, Ghanem, & Brooks, 2014), providing scientific basis for the development of value-added products.

The pharmacokinetics (PK) of an exogenous compound (drug or bioactive) characterize its fate in the body, after it is ingested, absorbed in the gastrointestinal tract, distributed by the systemic circulation, metabolized, and finally excreted. In the case of ACNs, a considerable body of evidence has demonstrated that in addition to the small intestine, the stomach (via bilitranslocase carrier) could have a role in their absorption (McGhie & Walton, 2007), and the retention of ACNs in these organs could contribute to their absorption and bioavailability. Bioavailability refers to the fraction of the compounds obtained through diet that reaches its site of action, i.e. the amount of compound ingested, adsorbed, and distributed that will reach the systemic circulation and the specific organs or tissues, which will later result in a biological effect (Degerud, Manger, Strand, & Dierkes, 2015). In addition, it has been also demonstrated that repeated exposure (and not higher concentrations) improves ACN bioavailability. For instance, Ferruzzi et al. (2009) showed that the AUC of some compounds improved up to 300 % compared to a single acute dose treatment using a mouse model of Alzheimer's disease receiving daily intra-gastric gavage of grape seed polyphenolic extract. For these reasons, an encapsulate that can extend ACN retention and promote a sustained release in the upper portions of the gastrointestinal (GI) tract is expected to improve ACN bioavailability.

Different strategies have been described in the pharmaceutical literature for the retention of dosage forms and drugs in the stomach, which could be applied for the development of an ACN encapsulate. For example, floating delivery systems (FDS) could be used by the food industry for the development of such encapsulates, as they often contain ingredients approved for food applications and do not require the use of solvents (Davis, 2005). These encapsulates could also prevent ACN degradation due to environmental and *in vivo* conditions. Up to this point, there has been little to no exploration of floating systems for increasing the retention of ACNs in the upper GI tract.

Therefore, the overall goal of this project was to develop a platform for oral delivery to prolong the residence time of ACNs extracted from haskap berries in the upper GI tract (mainly stomach), and modulate their release and absorption. To achieve this aim, the research objectives were to:

i) Investigate the extraction of ACNs from haskap berries using ultrasound-assisted extraction (UAE) and to determine the significant factors and optimal conditions;
ii) Develop two novel floating delivery systems (FDS) that could increase the retention of ACNs in the stomach: (1) floating microspheres and (2) an *in situ* gelling raft system;
iii) Determine the (1) physicochemical characteristics and (2) release profile of ACNs for both types of floating systems;
iv) Evaluate and compare the two floating systems to determine the most suitable option for further development.

The outline of this thesis follows a format where subsequent chapters are presented in manuscript form. In these chapters, evidence from the literature supporting the approach proposed in the thesis is presented. This is followed by the experimental work, research results, conclusions, and recommendations.

Chapter 2 is a literature review of the bioactive content and health benefits associated with haskap berries. The different methods used for quantification of antioxidant capacity are critically reviewed, and their limitations are presented. In

addition, current commercial products and the potential use of encapsulation technology for the development of value-added products are discussed. **Chapter 3** outlines the different encapsulation methods for various bioactive components used by the food industry. The methods presented are those from which a powder (or solid material) is directly obtained or requires additional steps for its production. The characteristics of the encapsulated materials that are relevant for the food industry in the view of potential applications are also summarized.

Chapter 4 reviews the current knowledge of the PK of ACNs (i.e. absorption, distribution, metabolism, and excretion). This information is used as the basis to propose a theoretical physiologically-based, multi-compartmental pharmacokinetic (PBPK) model to describe the fate of ACNs and their metabolites *in vivo*, which could be used for experimental design and interspecies scale-up. Additionally, this review recognizes the role played by the stomach in the absorption of ACNs, and its potential as a target for the development of a delivery system. **Chapter 5** presents gastroretentive systems (GRS) as a strategy to bridge the current gap between *in vitro* results that indicate ACNs are beneficial in managing degenerative diseases and evidence from clinical studies. Type 2 diabetes (T2D) is used as a model of degenerative disease, and potential causes for this gap are presented, followed by a discussion of delivery platforms that could be used to retain ACNs in the stomach for longer periods of time.

In **Chapter 6**, the ultrasound-assisted extraction of ACNs from haskap berries is investigated experimentally. The significant factors that impact the extraction are identified and optimized, using statistical methods for the design of experiments. In **Chapter 7**, the feasibility of encapsulating ACN-rich extract in calcium-alginate particles (a non-floating system) is demonstrated and experimental factors are optimized for a combined response for both encapsulation efficiency and size. **Chapter 8** describes the development and characterization of floating microspheres as a novel FDS to potentially increase the retention of ACNs in the stomach and to modulate their release. Here, the procedure established in Chapter 7 is further advanced to incorporate a gas-generating compound. Likewise, in **Chapter 9**, another novel strategy is proposed as GRS for ACN delivery and is based on an *in situ* gelling formulation to form a floating raft system. The potential implications for the administration of ACNs are also discussed. In **Chapter 10**,

the two FDS platforms (microspheres and *in situ* gelling raft system) are compared and evaluated. **Chapter 11** then concludes by providing a summary of all the research in this project with recommendations for future research.

Chapter 2 HASKAP BERRIES (*LONICERA CAERULEA* L.) - A CRITICAL REVIEW OF ANTIOXIDANT CAPACITY AND HEALTH-RELATED STUDIES FOR POTENTIAL VALUE-ADDED PRODUCTS

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Celli, G.B., Ghanem, A., & Brooks, M.S. (2014). Haskap berries (*Lonicera caerulea* L.) - a critical review of antioxidant capacity and health-related studies for potential value-added products. *Food and Bioprocess Technology*, *7*, 1541–1554.

2.1 ABSTRACT

Haskap berries commonly refer to fruits of *Lonicera caerulea* L., recognized by the Japanese aboriginals as the "elixir of life." Due to their recent arrival on the North American market, haskap berries have not yet been positioned among other berries and compared in terms of their phytochemical content. Haskap berries have higher ascorbic acid and anthocyanin content than other berries known for their health-promoting benefits, such as blueberries. In this article, we give a brief description of the phytochemical content and limitations of current methods used for their quantification, as well as a critical review of the techniques available to assess the antioxidant capacity of haskap extract in comparison to other berries. We then present results from recent studies with haskap extracts used in *in vitro* (bacterial and human cell cultures), preclinical trials (in animal models), and a first-in-human study to assess the implications for human health. Finally, we discuss current commercial products and the potential of encapsulation technologies to preserve the bioactivity and increase stability of the extracts, thus creating a new range of value-added haskap products.

2.2 INTRODUCTION

The plant kingdom is a valuable source of bioactive compounds, some of which have nutritional and other health-promoting properties for humans. Phytochemicals are plant metabolites that have been related to potential positive benefits to human health (Johnson, 2003). For example, it has been reported that in addition to exhibiting antioxidant activity, a phytochemical subgroup known as flavonoids also exerts modulatory effects on a variety of cells, acting on specific kinase-regulated pathways (Gamet-Payrastre et al., 1999; Matter, Brown, & Vlahos, 1992; Schroeter, Spencer, Rice-Evans, & Williams, 2001; Spencer, Rice-Evans, & Williams, 2003). Berries are a class of fruits that are particularly high in phytochemicals.

Recent reports bring attention to a fruit known in North America as haskap berries, blue honeysuckle, or honeyberries (*Lonicera caerulea* L.). These fruits are native to Siberia and northeastern Asia, where they are mainly found in low-lying wet areas or in mountains (Bors et al., 2012). In Russia, the first report as a horticultural plant was made in 1894 and attempts to domesticate *L. caerulea* L. occurred from 1913 (Hummer, 2006). Since the early 1950s, breeding programs in Russia have focused on the development of new cultivars with higher production, larger fruits, improved fertility, nutritional levels, and characteristics that ease the mechanical harvesting (such as even ripening) (Thompson, 2008). On the other hand, breeding programs in North America started only recently (Bors et al., 2012), due to the limited exchange of plant materials during this period (Thompson & Barney, 2007).

Currently, the haskap is not well-known in North America. However, some varieties and cultivars are already available in USA and Canada. Growers in Canada are using at least five different cultivars. In total, there have been over 35 Russian and 70 Japanese varieties identified (Bors et al., 2012).

Haskap, also known as “zhimolost” in Russia, mostly refers to the subspecies *kamtshatica*, *edulis*, and *boczkarnikovae*. In Japan, the term “haskap” or “hasukappu” is applied to *L. caerulea* var. *emphyllocalix* (Thompson, 2008). In Canada, the Japanese term “haskap” was maintained in reference to new breeding seedlings that do not share the negative characteristics of previous blue honeysuckle cultivars, such as bitterness

(Bors et al., 2012). The plant was traditionally used and recognized by Japanese Ainu aboriginals as "the elixir of life" (Thompson, 2006) for its therapeutic properties as an hypotensive agent (Anikina, Syrchina, Vereshchagin, Larin, & Semenov, 1988), reducing the effects of glaucoma and risk for heart attack, prevention of anemia, cure for malaria and gastrointestinal diseases, slowing the aging process, and providing elasticity to skin. In Hokkaido Island, a juice processor sells its product as a "gold remedy for eternal youth and longevity" (Lefol, 2007).

The purpose of this review is to present the current research on the chemical content and antioxidant capacity of haskap berries to justify a broader use of this fruit in health-related applications and briefly discuss the limitations of the analytical methods widely used for characterization and quantification of its phytochemicals. We also included results from *in vitro* and *in vivo* studies that support the use of these berries. Finally, we provide insights on future developments, based on the biochemical potential of these berries.

2.3 BOTANICAL DESCRIPTION

The genus *Lonicera* (Caprifoliacea family) encompasses approximately 200 species (Thompson, 2008) and is known for its ornamental bushes and vines with frequently inedible small yellow to red globose fruits. *L. caerulea* L. is an exception for its dark-blue to purple edible fruits (Bors et al., 2012). This long-lived shrub (Arus & Kask, 2007) is highly resistant to pests, diseases such as mildew, and soil types (Hummer et al., 2012). Haskap plants do not require special care during cultivation and could be an option for organic growers due to their immunity against pathogen infections (Hummer et al., 2012) and unnecessary application of fertilizers (Szot & Wieniarska, 2012). Another feature is the fruit cold hardiness. It has been reported that haskap shrubs can resist temperatures of −46 °C and the flowers can tolerate between −8 and −10 °C (Thompson, 2008). They are self-incompatible and in order to reproduce they require different cultivars and pollinators such as bumblebees (Hummer et al., 2012). Flowers accessible to pollinators generate 90 % of fruits, while isolated plants produce smaller and lighter fruits, with a lower number of seeds (Bożek, 2012).

Haskap shrubs are similar to blueberry and saskatoon berry shrubs in shape and size and thus could be grown using the same distribution spacing and harvested in a similar way. Blueberries require limited soil acidity (from pH 4.2 to 5.5), while haskap bushes tolerate a broader range of soil pH (from pH 5 to 8) (Retamales & Hancock, 2012). Haskap berries are more delicate and require less force to detach from the shrub, whereas Saskatoon berries are prone to pests and diseases. It has also been reported that haskap berries can float like cranberries, although studies applying the same harvesting techniques for both berries have not been performed (Bors et al., 2012).

Haskap fruit is generally a 2-cm elongated berry weighing 0.3 to 2.0 g, although the shape and weight depend on the variety. The dark skin of the fruit is covered by a waxy coating (bloom) and resembles the outer covering of blueberries and concord grapes (Arus & Kask, 2007; Bors et al., 2012; Hummer et al., 2012). Besides this similarity, haskap berries do not retain their shape once baked like most berries do (Bors et al., 2012). The flavor also varies, ranging from bitter to tart-sweet, which could suggest a mixture of known berry flavors (Hummer, 2006). The bitterness and acidity have been related to iridoid glucosides and esters of malic and citric acids isolated from the fruits (Anikina et al., 1988; Vereshchagin et al., 1989). Fruits are soft with tender skin and should be consumed or processed within 1 to 2 days; the firmer ones can be refrigerated up to a week (Ochmian, Grajkowski, & Skupień, 2008; Thompson, 2008).

Skupień, Ochmian, and Grajkowski (2009) and Ochmian et al. (2013) compared different Polish blue honeysuckle cultivars of early and late seasons. They concluded that the later fruits were significantly bigger (in diameter and length) and softer, with higher soluble solids and phenolic contents (especially anthocyanins) and lower titratable acidity and ascorbic acid concentration, possibly due to higher temperatures prior to the end of the season. This trend was also observed for mid-late and late ripening cultivars of apricot (*Prunus armeniaca* L.) (Mratinić, Popovski, Milošević, & Popovska, 2012), black currant (*Ribes nigrum* L.) (Krüger, Dietrich, Hey, & Patz, 2011), and apple (*Malus sylvestris* L.) (Warrington, Fulton, Halligan, & de Silva, 1999). Besides temperature, sunlight exposure has a significant role in the accumulation of some classes of phytochemicals and fruit composition, since it can influence the berry temperature. An increase in the fruit temperature as a consequence of direct exposure to sunlight for long

periods of time has negative effects on fruit color (Bergqvist, Dokoozlian, & Ebisuda, 2001). Although the taste was improved in the late season blue honeysuckle, the low firmness could affect handling and fruit quality for the commercial fresh market.

2.4 CHEMICAL CHARACTERIZATION

Research studies have been conducted to investigate the chemical composition and vitamin content of the haskap berry. Bors et al. (2012) believe that this berry could replace blueberries as a new "superfruit". Although haskap berries have great potential as a health-promoting fruit, it is critical that the analytical techniques used to determine the phytochemical content are robust and reliable, especially if the information is used to support health claims.

Ripening can significantly affect the physical (e.g. firmness and colour) and chemical (e.g. sugar and bioactive content) properties of fruits (Castellarin et al., 2016), which can impact postharvesting handling and their use in health-related products. As the ripening progresses, a series of events occurs leading to the dissolution of the middle lamella, weakening of parenchyma cell walls (Paniagua, Posé, Morris, Kirby, Quesada, & Mercado, 2014), degradation of hemicellulose, and modifications to the pectins, which will ultimately contribute to changes in texture (Huber, Karakurt, & Jeong, 2001). Later stages of ripening are characterized by physical growth, accumulation of sugars, and colour development (Castellarin et al., 2016; Stavang et al., 2015). Ochmian, Skupień, Grajkowski, Smolik, and Ostrowska (2012) recently reported changes in colour and firmess during the ripening of two haskap berry cultivars. There was a marked reduction of firmness (423±34 to 161±25 G/mm), puncture resistance (335±18 to 75±21 G/mm), and darkening of the fruits (from green and yellow to red and blue) as they ripened (Ochmian et al., 2012). Fully-ripened fruits have between 0.59±0.08 to 1.26±0.14 % of pectin, depending on the cultivar (Wojdyło, Jaúregui, Carbonell-Barrachina, Oszmiański, & Golis, 2013).

Vitamin C (ascorbic acid) is an essential water-soluble micronutrient in the human diet and recognized for its antioxidant activity, among several functions in biological systems. The recommended intake of ascorbate for a male adult is 90 mg/day (Food and

Agriculture Organization/World Health Organization [FAO/WHO], 2012; Naidu, 2003). The vitamin C content in haskap berries can vary from 30.5 to 186.6 mg/100 g fresh weight (FW) (Arus & Kask, 2007; Jurikova et al., 2012; Ochmian, Oszmianski, & Skupień, 2009; Palíková et al., 2008; Skupień, Oszmiański, Ochmian, & Grajkowski, 2007; Tanaka & Tanaka, 1998; Thompson, 2008), which is comparable to fruits with recognized vitamin content (Table 2.1). The ascorbic acid content in haskap berries is higher (by three- to ten-fold) than in blueberries, which are considered one of the richest sources of this compound (Harb, Khraiwesh, Streif, Reski, & Frank, 2010).

In terms of secondary metabolites, it appears that fertilizers and irrigation have no significant effects on the amount of *L. caerulea* L. metabolites, although the studies focusing on this subject are scarce (Pokorná-Juríková & Matuškovič, 2007; Szot & Lipa, 2012; Szot & Wieniarska, 2012). The phenolic group is very diverse, and these compounds are the main contributors to the antioxidant activity observed in many products. The total phenolic (TPC), flavonoid (TFC), and monomeric anthocyanin content (TAC) can be measured by spectrophotometric assays as previously described in the literature (Folin & Ciocalteu, 1927; Huang, Ou, & Prior, 2005; Lee, Durst, & Wrolstad, 2005; Lee, Rennaker, & Wrolstad, 2008; Stalikas 2007; Sondheimer & Kertesz, 1948).

The TPC of haskap berries assessed by Folin–Ciocalteu (FC) assay ranged from 140.5 up to 1,142.0 mg gallic acid equivalents (GAE) per 100 g of FW (Bakowska-Barczak, Marianchuk, & Kolodziejczykb, 2007; Fan, Wang, & Liu, 2011; Lefèvre et al., 2011; Palíková et al., 2008; Rop et al., 2011; Rupasinghe, Yu, Bhullar, & Bors, 2012; Skupień et al., 2007; Thompson & Chaovanalikit, 2003). These values are higher than those reported for other fruits (Table 2.2), such as red wild raspberry, black raspberry (Bakowska-Barczak et al., 2007), strawberry, physalis (Vasco, Ruales, & Kamal-Eldin, 2008), and blackberry (Acosta-Montoya et al. 2010). The results of TPC highly correlate to the antioxidant capacity tests based on reduction efficiency (Huang et al., 2005). Using the FC assay, Palíková et al. (2008) determined that the antioxidant capacity of haskap berries was 140.5 mg GAE/100 g FW.

Table 2.1 Vitamin C content (mg/100 g FW) in haskap berries and other fruit sources, where FW represents fresh weight

Fruit	Vitamin C (mg/100 g FW)	Reference
Haskap berry (*L. caerulea* L.)	30.5–186.6	Tanaka & Tanaka, 1998 Arus & Kask, 2007 Skupień et al., 2007 Palíková et al., 2008 Thompson, 2008 Ochmian et al., 2009 Jurikova et al., 2012
Watermelon (*Citrullus lanatus* Thunb. Mansfeld)	13.1–20.4	Tlili et al., 2011
Kiwi fruit (*Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Fergunson)	29.0–80.0	Nishiyama et al., 2004 Nunes-Damaceno, Muñoz-Ferreiro, Romero-Rodríguez, & Vázquez-Odériz, 2013
Raspberry (*Rubus idaeus* L.)	16.8–37.7	Pantelidis, Vasilakakis, Manganaris, & Diamantidis, 2007
Blackberry (*Rubus fructicosus* L.)	14.3–17.5	Pantelidis et al., 2007 Szajdek & Borowska, 2008
Red currant (*Ribes sativum* L.)	25.6–40.0	Pantelidis et al., 2007
Cranberry (*Vaccinium macrocarpum* Ait.)	11.5	Licciardello, Esselen, & Fellers, 1952
Strawberry (*Fragaria* x *ananassa* Duch)	23.8–51.0	Roberts & Gordon, 2003 Szajdek & Borowska, 2008
Orange (*C. sinensis* (L.) Osbeck)	54.0	Szeto, Tomlinson, & Benzie, 2002
Blueberry (*Vaccinium corymbosum* L.)	12.4–13.1	Szajdek & Borowska, 2008

Table 2.2 Total phenolic content (mg GAE/100 g FW) in haskap berries and other fruit sources. TPC, GAE and FW represent total phenolic content, gallic acid equivalents and fresh weight, respectively

Fruit	TPC (mg GAE/100 g FW)	Reference
Haskap berry (*L. caerulea* L.)	140.5–1,142.0	Thompson & Chaovanalikit, 2003 Bakowska-Barczak et al., 2007 Skupień et al., 2007 Palíková et al., 2008 Fan et al., 2011 Lefèvre et al., 2011 Rop et al., 2011 Rupasinghe et al., 2012
Red wild raspberry (*Rubus idaeus* L.)	120.8–455.5	Bakowska-Barczak et al., 2007 Benvenuti, Pellati, Melegari, & Bertelli, 2004 Deighton, Brennan, Finn, & Davies, 2000 Kalt, Forney, Martin, & Prior, 1999
Black raspberry (*Rubus neglectus* Peck)	411.8–980.0	Bakowska-Barczak et al., 2007 Wada & Ou, 2002
Strawberry (*F. ananassa* Duch)	86.4–238.0	Kalt et al., 1999 Vasco et al., 2008
Goldenberry (*P. peruviana* L.)	6.45–87.0	Valdenegro, Fuentes, Herrera, & Moya-León, 2012 Vasco et al., 2008
Blackberry (*Rubus adenotrichus* Schltdl.)	169.4–520.0	Acosta-Montoya et al., 2010 Zielinski et al., 2015

Within the phenolic group, flavonoids are a diversified class of compounds with more than 4,000 structures identified in plants to date (Whiting, 2001). The overall content of flavonoids is commonly assessed by the aluminum chloride ($AlCl_3$) spectrophotometric assay (Stalikas, 2007). Using quercetin as standard, Rupasinghe et al. (2012) found that the TFC of haskap berries varied from 54.7 to 699.3 mg quercetin equivalents (QE)/100 g FW, whereas blueberry (343.0 mg QE/100 g), strawberry (54.7 mg QE/100 g), and raspberry (63.5 mg QE/100 g) had the lowest values. Rop et al. (2011) used rutin as standard and the average content was 353.6 mg/100 g FW. Besides the standard used for quantification, the main differences between these studies were the amount of extract and reagents used as well as the solvent system (Rop et al., 2011; Rupasinghe et al., 2012).

Anthocyanins belong to the flavonoid group and are important pigments in higher plants, being responsible for the color of flowers and fruits (Castañeda-Ovando et al., 2009). The TAC measured by pH-differential method in haskap fruits is on average 1,300 mg cyanidin 3-glucoside equivalents per 100 g FW (369 mg/100 g FW when quantified as cyanidin 3-galactose equivalents) (Bakowska-Barczak et al., 2007; Fan et al., 2011; Lefèvre et al., 2011). This average anthocyanin content for haskap berries is higher than other fruit sources such as raspberry (Chen, Xin, Zhang, & Yuan, 2013), blackberry, red currant (Pantelidis et al., 2007), and blueberry (Skrede, Wrolstad, & Durst, 2000) (Table 2.3).

The spectrophotometric assays to determine the overall content of the classes of compounds described above have been widely applied to food and beverage matrices. It is worth mentioning that apart from the pH-differential method for anthocyanin, these procedures have low specificity for phenolics and flavonoids and can lead to an erroneous estimation of their content due to interferents commonly present in fruit—ascorbic acid, for example. Questions have arisen regarding the biological relevance of aluminum for the quantification of flavonoids, for example (Cornard & Merlin, 2002). Indeed, these assays are cheap, do not require elaborate equipment or procedures, and can be used as an indicator of the total amount of these compounds. However, confirmation with more robust techniques such as high-performance liquid chromatography (HPLC) is highly recommended. As a sole exception, the pH-differential method for determination of TAC

is approved by the Association of Official Agricultural Chemists (AOAC Official Method 2005.02) due to its good correlation with HPLC results (Lee et al., 2008).

Table 2.3 Total anthocyanin content (mg C3G/100 g FW) in other fruit sources. TAC, C3G and FW represent total anthocyanin content, cyanidin 3-glucoside equivalents and fresh weight, respectively

Fruit	**TAC (mg C3G/100 g FW)**	**Reference**
Haskap berry (*L. caerulea* L.)	1,300.0	Bakowska-Barczak et al., 2007 Lefèvre et al., 2011
Raspberry (*Rubus idaeus* L.)	22.2–436.9	Chen et al., 2013
Blackberry (*Rubus fructicosus* L.)	125.6–152.2	Pantelidis et al., 2007
Red currant (*Ribes sativum* L.)	1.4–7.8	Pantelidis et al., 2007
Blueberry (*V. corymbosum* L.)	99.9	Skrede et al., 2000

The phenolic compounds identified in haskap berries using more robust techniques are shown in the following table (Table 2.4). Palíková et al. (2008) stated that the anthocyanin and phenolic fractions in fresh haskap berry represent 0.31 and 0.4 %, respectively. On the other hand, Ochmian et al. (2009) demonstrated that Polish blue honeysuckle berries had lower anthocyanin (0.16 %) and total phenolic content (0.21 %) than those from Czech Republic (Palíková et al., 2008). Cyanidin 3-glucoside is the main anthocyanin, accounting for 80 to 92 % of the overall anthocyanin content (Jordheim, Giske, & Andersen, 2007; Palíková et al., 2008; Kusznierewicz et al., 2012). It is not surprising since this compound is the most widespread anthocyanin in nature (Kong, Chia, Goh, Chia, & Brouillard, 2003).

Table 2.4 Phenolic compounds identified in haskap berries

Anthocyanins	Flavonoids	Phenolic acids
Cyanidin 3-glucoside [a,b,c,d,e,f]	Quercetin 3-glucoside[b,d,f]	Chlorogenic acid[d,f]
Cyanidin 3,5-diglucoside[a,b,d,f]	Quercetin 3-rutinoside (rutin)[b,d,f]	Neochlorogenic acid[d,f]
Cyanidin 3-galactoside[a]	Quercetin-rhamnoside (quercitrin)[b]	Gallic acid[b]
Cyanidin 3-rutinoside[a,b,d,e,f]	Quercetin 3-galactose[d]	Hydroxycinnamic derivative (caffeic or ferulic acid)[f]
Cyanidin 3-xyloside[a]	Catechin[b]	4-aminobenzoic acid[b]
Delphinidin 3-glucoside[b]	Epicatechin[b]	3-caffeoylquinic acid[d]
Delphinidin 3-rutinoside[b]	Luteolin 7-glucoside[d]	5-caffeoylquinic acid[d]
Delphinidin 3-arabinoside-hexoside[b]		
Pelargonidin 3-glucoside[a,b,d,f]		
Pelargonidin 3,5-diglucoside[b]		
Pelargonidin 3-rutinoside[b]		
Peonidin 3-glucoside[a,b,d,f]		
Peonidin 3-rutinoside[b,f]		
Peonidin 3,5-diglucoside[b]		
Petunidin 3-glucoside[b]		

Note: Origin and varieties / cultivars identified as follow:

[a] Canada – n.s. (Bakowska-Barczak et al., 2007)

[b] Czech Republic – *kamtschatica, edulis*, "Goluboje vereteno", "Altaj" (Gazdik et al., 2008b)

[c] Norway – "Kirke" (Jordheim et al., 2007)

[d] Poland - *edulis* ("Wojtek", "Zielona", "Brazowa", "Jolanta", "46", "44") (Kusznierewicz et al. 2012; Ochmian et al., 2009)

[e] Russia – n.s. (Deineka, Sorokopudov, Deineka, Shaposhnik, & Kol'tsov, 2005)

[f] USA - *boczkarnikovae, edulis, stenantha, pallassi, kamtschatica* (Chaovanalikit, Thompson, & Wrolstad, 2004)

HPLC coupled with electrochemical detection (ED) was used for the identification of neuroprotective substances in haskap extracts (Gazdik et al., 2008b). HPLC–ED could also be a potential technique for elucidating antioxidant compounds in fruit extracts (Gazdik et al., 2008a). Another strategy is the use of gas chromatography

(GC) coupled with mass spectrometry (MS) detection as described by Zadernowski, Naczk, and Nesterowicz (2005) for the evaluation of bonded and free phenolics of haskap berries. It has been shown that haskap berries have less free phenolic acids than blueberries and blackberries (1.7, 2.6, and 3.3 %, respectively), but higher content of compounds liberated from esters (62.3, 40.7, and 53.1 %, respectively).

2.4.1 Antioxidant Capacity Measurements

Free radicals have been identified as the cause of some cancers and chronic diseases, and it is not surprising that research into phytochemicals that could act as antioxidants has increased in the past years. Several methods have been developed to assess the antioxidant capacity of plant extracts based on different mechanisms; however, there is no universally accepted standard assay (Tsao & Deng, 2004). The lack of a standardized assay can mislead the comparison and comprehension of results between research groups and the general public (Huang et al., 2005). Examples of methods include oxygen radical absorbance capacity (ORAC) (Ou, Hampsch-Woodill, & Prior, 2001), FC assay, Trolox-equivalent (TE) antioxidant capacity (TEAC), 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) (Apak et al., 2013; Huang et al., 2005). A different group of assays is applied when investigating the antioxidant activity of biologically relevant oxidants, including singlet oxygen, superoxide anion, and hydroxyl radical methods (Huang et al., 2005). In the following sections, *in vitro* assays for assessing antioxidant capability in haskap berries are briefly described and discussed.

2.4.1.1 Oxygen Radical Absorbance Capacity

The ORAC assay was first proposed by Cao, Alessio, and Cutler (1993) and is based on the reduction of fluorescent emission as a result of chemical changes on fluorescein (probe) by free radicals (Glazer, 1990; Ou et al., 2001). The method uses a hydrophilic vitamin E analog (Trolox, 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) as standard (Cao et al., 1993).

ORAC values for haskap berries range from 18 to 262.4 μmol TE/g of fresh berries (Chaovanalikit et al., 2004; Rop et al., 2011; Thompson & Chaovanalikit, 2003). From the same study, Rupasinghe et al. (2012) identified that some cultivars have higher values than blueberries (*Vaccinium angustifolium* Ait.), blackberries (*Rubus fruticosus* L.), strawberries (*Fragaria ananassa* Duch), and raspberries (*Rubus idaeus* L.), which could be associated with a higher antioxidant capacity.

Besides the lack of information, one of the main issues with ORAC is the difficulty in correlating the values with biological effects. For this reason, the ORAC table was removed from USDA Nutrient Data Laboratory (NDL) in 2010. It also commonly applies a larger amount of probe than antioxidant, which does not reflect the true proportion present in the organism.

2.4.1.2 α,α-Diphenyl-β-Picrylhydrazyl Radical Scavenging Activity

The DPPH assay is a rapid, inexpensive, convenient, and accurate method to evaluate antioxidant activity (Blois, 1958; Brand-Williams, Cuvelier, & Berset, 1995; Papariello & Janish, 1966; Sharma & Bhat, 2009). DPPH solutions have a violet color, and the delocalized unpaired electron is responsible for the strong absorption at 517 nm. Reduction reactions can be seen as decolorization (Blois, 1958; Molyneux, 2004). Results can be expressed as EC_{50} (effective concentration of antioxidant that reduces 50 % of the DPPH radical) or percentage of decolorization (Molyneux, 2004).

Raudsepp et al. (2013) showed that haskap berry extracts reduce up to 85 % of the DPPH radical amount, more than tomato (*Lycopersicon esculentum* Mill.) (average 43 %), sea buckthorn (*Hippophae rhamnoides* L.) (74 %), and the standard ascorbic acid (average 51 %). It was also reported that purified haskap berry extracts have higher antioxidant capacity represented by lower EC_{50} values (30 μg/mL) than bog bilberry (*Vaccinium uliginosum* L., 35 μg/mL) and raspberry extracts (*R. idaeus* L., 50 μg/mL) (Zhao et al., 2011). Rupasinghe et al. (2012) also reported high antioxidant capacity of haskap berry crude extract: EC_{50} was on average 6.7 mg FW/mL compared to 32.27 mg FW/mL for blueberry (*V. angustifolium* Ait.). Although both studies agree with the high antioxidant capacity of haskap extract, they differ in terms of units used to express the

results (μg/mL and mg/mL, respectively). Besides the method and equipment used, a possible explanation for this difference is the extract preparation, since Zhao et al. (2011) included additional purification steps to evaluate the antioxidant activity of anthocyanins in the purified extract, whereas Rupasinghe et al. (2012) evaluated the activity in crude extract without a purification step. Rop et al. (2011) indicated that EC_{50} values range from 76 to 134 μg/mL in haskap berry crude extract. Results expressed in ascorbic acid equivalents (AAE) were higher for haskap berries (6.59–10.17 g AAE/kg FW) than cherries (*Prunus avium* L., up to 0.9 g AAE/kg FW) or plums (*Prunus domestica* L., up to 6 g AAE/kg FW) (Rop et al., 2011).

Some concerns arise when evaluating the appropriateness of the DPPH method to predict outcomes *in vivo*. For example, unlike reactive species, the DPPH is a long-lived radical, and it is likely that the antioxidant response of biologically relevant antioxidants is different from that with the DPPH radical.

2.4.1.3 Trolox-Equivalent Antioxidant Capacity

Developed by Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993), TEAC is also a colorimetric assay based on the reduction of bluish-green 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cations (Miller et al., 1993). Zhao et al. (2011) once again demonstrated the higher antioxidant capacity of haskap extract when compared to bog bilberry (*V. uliginosum* L.) and raspberry (*R. idaeus* L.). Results also show the higher antioxidant capacity of the haskap berry (9.55 mmol TE/100 g FW) compared to bilberry (*Vaccinium myrtilloides* Michx., 4.11 mmol TE/100 g), red wild raspberry (*R. idaeus* L., 3.23 mmol TE/100 g), and wild strawberry (*Fragaria virginiana* Duch., 1.95 mmol TE/100 g) (Bakowska-Barczak et al., 2007).

Some authors reported that the TEAC method underestimates the antioxidant capacity of complex foods when compared to ORAC assay (Zulueta, Esteve, & Frígola, 2009) due to incomplete scavenging during the reaction time (Arts, Haenen, Voss, & Bast, 2004b; Tian & Schaich, 2013). It has been suggested that changing the ABTS radical concentration could at least attenuate this problem (Arts, Dallinga, Voss, Haenen,

& Bast, 2004a). Also, substances that can absorb light at a wavelength of 734 nm can interfere with the results (Apak et al., 2013).

2.4.1.4 Ferric Reducing Antioxidant Power

The FRAP assay was introduced by Benzie and Strain (1996) and is based on a change in the oxidation state of iron under low pH. The values of FRAP for haskap berries range from 7.57 to 113 μmol of TE/g of fresh weight (Rop et al., 2010; Sánchez-Moreno, 2002). Some haskap cultivars have higher values than blueberry (*V. angustifolium* Ait., 16.24 μmol TE/g), blackberry (*R. fruticosus* L., 15.03 μmol TE/g), strawberry (*F. ananassa* Duch., 8.0 μmol TE/g), and raspberry (*R. idaeus* L., 7.57 μmol TE/g) (Rupasinghe et al., 2012). These differences are associated with the reducing potential of compounds present in the haskap extract.

The pH 3.6 required for this analysis improves iron insolubility, although it could decrease the ionization potential of some compounds needed for the electron transfer reaction. Generally, antioxidant capacity measured by FRAP is poorly correlated to other assays.

2.4.1.5 Hydroxyl Radicals (•OH) Scavenging Method

Hydroxyl radicals (•OH) are highly reactive oxidized species and are formed by the Fenton reaction (Lloyd, Hanna, & Mason, 1997). These hydroxyl radicals do not last long after the reaction, and the relevance of this methodology *in vivo* is questionable (Huang et al., 2005). Zhao et al. (2011) evaluated the hydroxyl radical scavenging capacity of haskap berry anthocyanin extract in comparison to an ascorbic acid positive control. The concentration of anthocyanins in the extracts had a positive correlation with the scavenging capacity. In general, *L. caerulea* L. had a higher capacity than *V. uliginosum* L. and *R. idaeus* L. (Zhao et al., 2011). Rop et al. (2011) used a slightly modified method to evaluate the hydroxyl scavenging capacity. The results of their methanolic extract ranged from 26 to 37 % of hydroxyl radicals scavenging (Rop et al., 2011), which was lower than the average 70 % reported by Zhao et al. (2011). The

scavenging capacity of the haskap berry is comparable to that reported for blackberry (*Rubus sp*.) (72 %) and higher than some varieties of cranberry (*Vaccinium macrocarpon* Ait.) (64.2 %), raspberry (*R. idaeus* L. and *Rubus occidentalis* L.) (66.9 %), blueberry (*Vaccinium spp.*) (58.7 %), and strawberry (*F. ananassa* Duch.) (68.6 %) (Wang & Jiao, 2000).

2.4.1.6 Superoxide Anion Radicals ($O_2^{\bullet -}$) Scavenging Method

The method used to evaluate superoxide scavenging measures the capacity of compounds to inhibit radical formation on a hypoxanthine and xanthine oxidase system (Sánchez-Moreno, 2002). It has been shown that the superoxide scavenging capacity of a haskap phenolic fraction to inhibit 50 % of NADH oxidation is 115 μg phenolics/mL (Palíková et al., 2008). Rop et al. (2011) evaluated the superoxide scavenging capacity of haskap berry extracts using an enzymatic system containing xanthine oxidase, xanthine, and cytochrome c as a probe. The authors reported a higher scavenging activity of haskap berries (41.15 % scavenging) than the methanolic extract of black chokeberry cultivars (*Aronia melanocarpa* (Michx.) Elliot, which was on average 39 %) (Rop et al., 2010). Both scavenging methods are not specific to phenolics, and other reducible species that are present can interfere with the results.

2.4.1.7 HPLC–ED and Post-Derivatization Column for Antioxidant Potential Determination

As mentioned previously, HPLC coupled with ED could be applied to determine the "antioxidant potential" of a complex mixture. Even though it does not measure a true antioxidant capacity – no reactive species are used – it is possible to correlate physicochemical characteristics of phenolics with their reduction and scavenging properties. Unlike previous methods, their structure is more important than the amount of polyphenolics (Mannino, Brenna, Buratti, & Cosio, 1998).

It is implied that the oxidation potential of phenolics is inversely proportional to the capacity to donate electrons and estimates the energy required for it. For example,

compounds with low oxidization potentials would have high antioxidant activity because it is easier to donate this electron to reactive species (Mannino et al., 1998). In their study, Mannino et al. (1998) found a good correlation between electrochemical potential and phenolics quantified by the FC method for white wines. In red wines, phenolics found in small amounts have higher antioxidant activity leading to a low correlation between the methods. Guo, Cao, Sofic, and Prior (1997) also found a good correlation between HPLC–ED and ORAC and reinforced that the former method could be used to identify other antioxidants, such as vitamin C. According to Peyrat-Maillard, Bonnely, and Berset (2000), when simple phenolics are the main compound responsible for antioxidant activity, the electrochemical potential can be correlated to this activity. However, they could not establish a good relationship for flavonoids due to their structural complexity (Kool et al., 2007). Gazdik et al. (2008a) used an HPLC–ED technique to identify flavonoids with antioxidant activity in haskap berries using the DPPH assay as a reference. Their results corroborate those obtained from other methods, providing further evidence of the high antioxidant capacity of *L. caerulea* fruits. The authors also reported a good correlation between HPLC–ED and DPPH methods (Gazdik et al., 2008a).

The use of a HPLC post-derivatization column has been also described for the identification of specific antioxidants in a complex mixture after chromatography separation (Kool et al., 2007). Kusznierewicz et al. (2012) evaluated the antioxidant activity of haskap berry extracts using a HPLC post-derivatization column with ABTS radical and FC reagent. The authors noted that antioxidant potential determined using ABTS yielded higher results than the FC reagent. They concluded that the anthocyanin is the main class responsible for the antioxidant activity of haskap berries (Kusznierewicz et al., 2012).

2.4.1.8 Summary of *In Vitro* Assessment of Antioxidant Capacity

Results from *in vitro* tests using haskap extracts are comparable or superior to other berries commonly referred to as “superfruits”. Different methodologies have been

used to demonstrate their possible antioxidant capacity. As there is no official method, this can make comparison of the results difficult.

The biological importance of *in vitro* tests to quantify antioxidant capacity has been questioned. The methods discussed in the previous sections are based on chemical reactions that occur *in vitro* under controlled conditions and are not able to properly simulate the human organism. The results from these *in vitro* tests do not provide sufficient information to draw conclusions about the health benefits associated with phytochemicals (Huang et al., 2005). Further analyses are necessary to justify the involvement of these plant metabolites on biological systems, and use of live cells is the next step when screening for possible activities.

2.5 HEALTH-RELATED BENEFITS ESTIMATED BY CELL CULTURE AND *IN VIVO* TESTS

Recent *in vitro* and *in vivo* studies have shown that the consumption of a variety of berries could be associated with a number of positive health benefits, including the reduction of cancer (Gordillo et al., 2009), improved hyperglycemia and insulin resistance (Stull et al. 2010; Vuong et al. 2009), lower bone loss in an ovariectomized rat model (Devareddy et al., 2008), and neurocognitive improvement (Krikorian et al., 2010). Some of the health benefits associated with haskap berries determined by *in vitro* and *in vivo* tests are presented in Table 2.5 and discussed in the following sections.

Table 2.5 Benefits associated with haskap berries determined by *in vitro* and animal model tests

Benefits	**Reference**
Antimicrobial properties, suppression of biofilm formation and adhesion	Palíková et al., 2008 Raudsepp et al., 2013
Inhibition of liver microsome peroxidation and delay of LDL oxidation	Palíková, Valentová, Oborná, & Ulrichová, 2009
Inhibition of melanogenesis	Misaki, Mizutani, & Itakura, 2006
Positive effect against UVA and UVB radiation	Svobodová, Zdařilová, & Vostálová, 2009 Zhao et al., 2012
Decrease of reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) on gingival fibroblasts	Zdařilová, Svobodová, Chytilová, Šimánek, & Ulrichová, 2010
Attenuation of eye inflammation on induced uveitis	Jin et al., 2006
Positive effects on unbalanced high-fructose diet	Jurgoński, Juśkiewicz, & Zduńczyk, 2013
Reduction of tumor volume	Gruia, Oprea, Gruia, Negoita, & Farcasanu, 2008

2.5.1 Results from Bacterial Cell Culture Tests

Based on bacterial cell culture tests, it has been shown that haskap berries have antimicrobial properties. Haskap berry extracts prepared by decoction with water are highly effective against *Bacillus subtilis*, *Kocuria rhizophila*, and *Campylobacter jejuni* growth, whereas ethanolic extracts show good results against *Escherichia coli*. The

haskap berry would be a good candidate as a probiotic food, since it has no negative effects on health-related bacteria (Raudsepp et al., 2013). Palíková et al. (2008) also demonstrated a significant effect of freeze-dried berries and its phenolic extract on reducing the adhesion of *Staphylococcus epidermidis* (0 colony forming units, CFU), *E. coli* (4.55×10^3 and 1.35×10^1 CFU, respectively), *Enterococcus faecalis* (6.9×10^3 and 4.5×10^0 CFU, respectively), and *Streptococcus mutans* (4.48×10^1 and 0 CFU, respectively) to an artificial surface compared to the respective controls (2.59×10^3, 1.6×10^4, 6.1×10^4, and 5.44×10^4 CFU). The adhesion is necessary for microbial colonization and infection of a host organism, especially for urinary and mouth diseases (Palíková et al., 2008).

2.5.2 Results from Human Cell Culture Tests

Lipid peroxidation can lead to cardiovascular diseases, such as atherosclerosis (Aruoma, 1998). The inhibition of liver microsome peroxidation (IC_{50} of 160 ± 20 μg/mL) and delay of LDL oxidation (up to 400 *vs*. 150 min for ascorbic acid) in cell culture has been achieved using a phenolic extract of haskap berries with 18.5 % of anthocyanins (Palíková et al., 2009).

In addition, haskap berry extracts could potentially be used in cosmetic applications as a whitening agent and also as a compound to act against the development of melanoma due to its inhibition effect on melanogenesis (melanin production). Haskap extracts also inhibited stem cell factor (SCF)-induced c-kit (receptor for SCF) phosphorylation, suppressed SCF-induced proliferation of melanocytes, and decreased their melanin content (Misaki et al., 2006). SCF present in keratinocytes activates the c-kit receptor in melanocytes, leading to melanogenesis (Uong & Zon, 2010).

A polyphenolic extract obtained from haskap berries was applied to a cell culture of gingival fibroblasts. It was shown that the haskap berry extract decreased ROS and TBARS (products of lipid peroxidation) concentrations. Fibroblasts also showed reduced lipid peroxidation, glutathione depletion, inflammatory markers release (interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF-α)), and COX-2 induction (Zdařilová, Svobodová, Chytilová, Šimánek, & Ulrichová, 2010).

2.5.3 Results from Animal Trials

Results from mice trials confirmed the radioprotection potential of haskap berries (Zhao et al., 2012). Phenolic extracts both in pre- and post-treatment significantly decreased UVA and UVB radiation effects, such as keratinocytes injury, DNA breakage, free radical production, membrane damage, and lipid peroxidation. Phenolic extracts also increased the concentration of reduced glutathione, which exhibits antioxidant activity. A decrease in caspase-3 and caspase-9 activity was also observed. Caspase proteins are related to apoptosis, a programmed cell death. The effects were associated with a mixture of compounds in the haskap extract (Svobodová et al., 2009).

Attenuation of inflammation was also observed in rat eyes with lipopolysaccharide (LPS) endotoxin-induced uveitis, a recognized model for acute ocular inflammation. Haskap berry extract was found to inhibit the nuclear factor-κB (NF-κB)-dependent signaling pathway that leads to the production of proinflammatory mediators, such as TNF-α (Jin et al., 2006).

Furthermore, positive effects were demonstrated in rats supplemented with an unbalanced high-fructose diet. Haskap berry extract containing 51.7 % of anthocyanins decreased plasma lipids other than HDL and normalized triglyceride concentrations. It also enhanced lactase activity and improved insulin resistance. Once again, the effects were observed for the whole berry extract and not for individual compounds. Based on the animal results, the authors calculated a theoretical daily dose of 0.8 g of phenolic extract for an adult (70 kg) that would promote similar benefits observed in rats (Jurgoński et al., 2013).

Haskap extracts have been related to the reduction of tumor volume in rats when administered during the growth and development stages. However, the authors also found some evidence for the promotion of tumor growth by the haskap extracts. As mentioned in their research paper, the extracts contained small amounts of peroxides, which could be an indication of contamination and the possible cause of the prooxidative effects (Gruia et al., 2008).

2.5.4 Results from Human Trials

Bacterial and mammalian cell cultures are useful techniques when predicting the health benefits, safety, and toxicity of plant-derived products. They are an important step during drug development, preceding preclinical studies with animal models and guiding the decision-making to stop or continue the research (Breslin & O'Driscoll, 2013). However, positive results from *in vitro* tests do not guarantee *in vivo* efficiency, and further tests on humans are required. The effects of phytochemicals on human health can only be observed once the compounds achieve their target in the body in a therapeutic concentration (Lundquist & Renftel, 2002). Although flavonoid metabolism has not been completely elucidated, it is supposed that the bioactive forms of flavonoids *in vivo* are not the same ones found in plants. For example, they could be conjugates and metabolites of glycosides and aglycones instead (Williams, Spencer, & Rice-Evans, 2004).

There has been, to our knowledge, only one recent clinical trial investigating the effect of haskap berry consumption on human health. Heinrich et al. (2013) evaluated the daily consumption of 165 g of fresh haskap berries (208 mg of anthocyanins per day) by healthy volunteers for a week. No changes in clinical, biochemical, and hematological tests and total antioxidant activity of plasma were observed in the volunteers. However, there was an increase in simple phenolics and hippuric acid concentration in urine as a result of anthocyanin metabolism. It also significantly augmented glutathione peroxidase, catalase in erythrocytes, and TBARS levels, which could suggest a minor antioxidant activity (Heinrich et al., 2013).

2.5.5 Summary of *In Vitro* and *In Vivo* Findings and Relevance for Human Health

Although some health benefits have been associated with haskap berries, those effects still need to be confirmed. There is a significant difference between the quality and quantity of phenolics found in fresh foods and after their absorption in the human body. Cell culture and animal model tests can help identify possible activities and mechanisms of action of phenolics; however, they do not provide all the answers needed to elucidate their health-promoting role. The study by Heinrich et al. (2013) conducted

with healthy volunteers is the first attempt to evaluate the effect of haskap berries on humans. Further analyses are needed in order to identify beneficial or harmful effects in volunteers consuming the berries for more than a week. In addition, trials on volunteers with nutritional deficiencies and/or specific diseases should be conducted.

2.6 COMMERCIAL PRODUCTS AND FUTURE DEVELOPMENTS

The potential of lesser-known fruits as functional foods and ingredients for nutraceuticals or pharmaceuticals has made it necessary to identify the compounds with the associated health benefits (Jurikova et al., 2012). The aim of this section is to briefly describe commercially available haskap products and to demonstrate how encapsulation technology can be used to create new value-added products. It is worth noting that the uniformity of ripeness is an important factor to be considered in the development of health-related products, as it can impact the concentration of bioactive compounds in the starting material (Stavang et al., 2015).

As haskap berries are extremely perishable and a seasonal crop, freezing or drying allows for long-term storage of the berries for the year-round production of commercial products. Even though freezing storage is the main process used for long-term preservation of fruit products, changes can occur during storage (Celli, Ghanem, & Brooks, 2015a). However, greater retention of bioactive compounds in the haskap berries can be achieved with steam blanching before frozen storage (Khattab, Celli, Ghanem, & Brooks, 2015a) and microwave thawing (Khattab et al., 2015b). A recent report by our group has also demonstrated the feasibility of Refractance Window™ drying for the production of a haskap berry powder with ~93.8 % retention of ACNs from the original frozen fruits (Celli, Khattab, Ghanem, & Brooks, 2016).

Haskap berries are currently marketed in jam, jelly, wine, candies, gelatin, puffed snacks (Liu, Zheng, Jia, Ding, & Gao, 2009; C. Liu et al., 2010), ice cream, and yoghurt. In Japan, the products are targeted to upper-middle and upper classes due to high costs associated with manual harvesting (Lefol, 2007). The berries are very suitable for juice processing, with a stable color that is not influenced by storage (Thompson, 2008) and

the effects of different sterilization methods on haskap juice have been previously described (Piasek et al., 2011).

As outlined in earlier sections, research studies have indicated health-promoting benefits from the consumption of haskap berries and extracts. Processing techniques that allow encapsulation of the berry extract in a protective wall matrix are a potential means of creating a value-added product with the benefits of controlled release and increased stability of the extract. Encapsulation technology can create high-value products for the nutraceuticals market, as they can be designed to allow efficacious concentrations of health-promoting compounds to reach the target organs, thus increasing their bioavailability and long-term stability (Aqil, Munagala, Jeyabalan, & Vadhanam, 2013). There are many different encapsulation methods and materials that can be used for polyphenolic compounds, and the reader is referred to the review by Munin and Edwards-Lévy (2011) and Celli, Ghanem, and Brooks (2015b) for specific details, as it is beyond the scope of the current article. Results from selected studies on the encapsulation of other berry extracts are described in the following paragraphs to illustrate the value-added benefits of encapsulation.

In a study by Zheng, Ding, Zhang, and Sun (2011), polyphenolic bayberry extract encapsulated in ethyl cellulose microparticles presented a higher antioxidant capacity, measured as hydroxyl and DPPH scavenging capacity, compared to the non-encapsulated extract. This activity was also preserved after storage for a week. The microcapsules were also found to be stable under different environmental conditions, such as light, heat, and moisture, measured as polyphenolic retention rate. Here, the release rate of polyphenols reached up to 87 % in *in vitro* tests simulating intestinal fluid (pH 8.0).

Blueberry extracts encapsulated with maltodextrin were also found to preserve the antioxidant capacity, phenolic, and anthocyanin content, where encapsulation was achieved through spray drying and varying the ratio of extract to maltodextrin, and inlet and outlet temperatures (Ma & Dolan, 2011). The stability of blueberry anthocyanins was found to be improved after encapsulation in nanoparticles produced with bovine serum albumin (BSA) and under neutral pH (Chen, Tao, Zhang, Sun, & Zhao, 2014). Storage stability was also improved in phenolic cloudberry microencapsulates produced with

maltodextrin by freeze drying that also resulted in the maintenance of the antioxidant capacity (Laine, Kylli, Heinonen, & Jouppila, 2008).

Based on the examples described from the literature, encapsulation technology has the potential to extend the shelf-life of haskap berry polyphenolic extracts. In addition, the design of controlled-release delivery systems for therapeutic applications and the use of an encapsulated extract powder as a nutraceutical ingredient for incorporation into functional foods are exciting possibilities for the future.

2.7 CONCLUSIONS

Haskap berries have been fairly unknown in North America until recently. Research studies have shown that haskap berries are a promising source of health-promoting phytochemicals. In the past years, an increasing number of research studies have been focusing on the characterization and quantification of these phytochemicals from haskap berries and the potential benefits associated with their consumption. Different methods can be applied to assess the biological relevance of the haskap berries.

Phenolic compounds such as flavonoids and anthocyanins have been correlated to the antioxidant activity exhibited by berries. TPC is widely determined by the Folin–Ciocalteu assay, which lacks in specificity. The quantification of TFC is based on the complexation with $AlCl_3$, an unspecific reaction that can be interfered with by other classes of compounds. Unlike previous methods, the pH-differential method used for the quantification of TAC is robust and presents high correlation with HPLC analysis.

Results from *in vitro* tests using haskap extracts have shown that these berries have comparable or superior antioxidant capacity than fruits commonly referred to as "superfruits". However, the comparison of results is difficult due to the lack of a standardized method to assess antioxidant capacity. The extrapolation of the results to biological systems is questionable as most radicals and the controlled conditions used in these assays are not present in and do not simulate the human organism. At present, *in vivo* assays cannot yet confirm the results from *in vitro* tests with haskap berries.

Possible health benefits associated with the consumption of haskap berries have also been investigated. Although the clinical studies in human volunteers are limited,

results from *in vitro* studies and animal models can help justify a broader use of these fruits. Future research studies are necessary to elucidate their effects on biological systems.

Studies on the effects of processing conditions on haskap berries and extracts are also needed in order to preserve and maximize the phytochemical content in potential functional foods and pharmaceutical products. Encapsulation delivery systems emerge as a powerful method to deliver bioactive haskap compounds to target organs and tissues, as well as improve long-term stability.

Chapter 3 BIOACTIVE ENCAPSULATED POWDERS FOR FUNCTIONAL FOODS - A REVIEW OF METHODS AND CURRENT LIMITATIONS

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Celli, G.B., Ghanem, A., & Brooks, M.S. (2015). Bioactive encapsulated powders for functional foods – a review of methods and current limitations. *Food and Bioprocess Technology*, *8*, 1825–1837.

3.1 ABSTRACT

The incorporation of functional ingredients in food products is often limited by their taste and instability during processing and storage. Encapsulation techniques are commonly used to overcome these limitations and for the development of value-added products. Although different methods and materials are available, their selection will depend on the bioactive properties and the desired characteristics of the encapsulate and the final product. Physicochemical characteristics of encapsulates should be assessed and considered during all stages of food processing. Although a considerable body of literature has reported the encapsulation of bioactive compounds, there is still a large gap between research and their application and commercialization into food products. The purposes of this review are to provide an overview of the current research on the encapsulation of bioactive components and methods used for their preparation, discuss relevant physicochemical characteristics that should be considered for the application of encapsulates into food products, and provide directions for future research.

3.2 INTRODUCTION

Research has provided evidence that supports the use of foods as active agents in the prevention or amelioration of nutrition-related diseases and improvement of the quality of life (Oyebode et al., 2014). This has contributed to an increased consumer

demand for functional foods (Day, Seymour, Pitts, Konczak, & Lundin, 2009), which are foods that have been enhanced with bioactive ingredients and have demonstrated health benefits beyond basic nutrition. The current functional food market can be categorized into the following main groups: dairy products (43 %), bars and cereals (20 %), beverages (15 %), fats and oils (12 %), and bakery products (2 %) (Sanguansri & Augustin, 2010). The incorporation of typical bioactive compounds (e.g. vitamins, lipids, and peptides) and probiotic cells in different food matrices is limited by their inherent characteristics, such as taste and incompatibility with the formulation and its ingredients, and subsequent processing that can affect the retention of labile compounds (Augustin & Hemar, 2009). In this respect, encapsulation is a versatile technology that can address these market needs, contributing to the development of high-value functional foods.

Encapsulation is the process used to entrap one substance (termed core material or active agent) within another (coating, shell, or carrier/wall material). Encapsulation techniques can contribute to extending the product's shelf life, protecting the active components against degradation during storage and maintaining their functionality; masking unwanted flavour, smell, or taste (Luca, Cilek, Hasirci, Sahin, & Sumnu, 2014); and increasing the effectiveness of natural functional compounds that normally have a lower potency at equivalent levels when compared to synthetic ingredients (Yallapu, Gupta, Jaggi, & Chauhan, 2010).

Encapsulates can be arbitrarily classified into two main categories: reservoir and matrix (or monolithic) types, although a combination of both systems is also possible (Zuidam & Shimoni, 2010). Reservoir encapsulates consist of an outer shell surrounding the active substance and can be formed by one or multiple reservoir chambers. In matrix systems, the active agent is in the form of droplets or dispersed in the encapsulating material (Zuidam & Shimoni, 2010). Depending on the technique and matrix chosen, some of the bioactive compounds can be exposed on the surface of the particles resulting in incomplete encapsulation, which makes this type of approach unsuitable for certain compounds (Tamjidi, Nasirpour, & Shahedi, 2012).

Although numerous techniques have been described in the literature, their selection will depend on the characteristics of the bioactive components (molecular weight, polarity, etc.) and, consequently, the desired physicochemical properties of the

encapsulate, such as solubility, partitioning, and stability. In addition to the usual characteristics exhibited and shared with other food powders (e.g. particle size, surface morphology, etc.), the encapsulate should contain a significant amount of the bioactive in a form that can be easily incorporated in food matrices, protect the core material against degradation (throughout the food processing chain and *in vivo*) ensuring that it will remain in its active form, and be compatible with the food matrix in which it is applied. Other conditions should also be considered when selecting the appropriate technique and materials for the development of encapsulated powders, such as the time, cost, and steps required for their preparation, production volume, market requirement, and regulations (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011).

The purpose of this review is to highlight the importance of the physicochemical properties of bioactive powder encapsulates for their application in functional foods and beverages. First, the methods and some materials used for the preparation of encapsulated powders are briefly reviewed, followed by an overview of the current research on the encapsulation of bioactive components and some examples of their incorporation in food products. Then, a discussion of relevant physicochemical characteristics that should be considered for the application of encapsulates into food products is provided. In this review, we focus on microencapsulates used in their powder form, although different types have been developed, including solutions, suspensions, and gels.

3.3 ENCAPSULATION TECHNIQUES

Several techniques have been described in the literature for the encapsulation of bioactive components. Due to the large number of methods currently used in the field, in this review they will be grouped based on those from which a powder is directly obtained and the ones that require an additional step for the production of the powder. Table 3.1 provides a summary of some techniques used by the food industry, which are briefly presented in the following section.

Table 3.1 Summary of some encapsulation techniques used by the food industry and characteristics of the encapsulates produced (adapted from Zuidam & Shimoni 2010)

Process	Type	Load (%)	Particle size (μm)	Advantages	Disadvantages
Methods from which a powder is directly obtained					
Spray drying	Matrix	5–50	10–400	- Uniform product when the appropriate procedure is used - Easy to operate and scale up - Operate continuously - For heat-sensitive compounds - Cost-effective	- Installation costs - Incomplete encapsulation - Micro-cracks on the surface - Low thermal efficiency
Spray cooling/chilling	Matrix	10–20	20–200	- Secondary coating for controlled release - Cost-effective - Operated continuously - Easy to operate and scale up	- Lipophilic coating (water-insoluble particles) - Expulsion of bioactive during storage
Freeze or vacuum drying	Matrix	Various	20–5,000	- For heat sensitive compounds - High retention of volatiles - Rapid and nearly complete rehydration of the dried product	- Sample has to be frozen (in the case of freeze drying) - For water-soluble compounds - High energy use - Long processing time (> 20 h) - Porous structure

Process	Type	Load (%)	Particle size (μm)	Advantages	Disadvantages
Methods that require additional step for the production of powder					
Fluid bed coating	Reservoir	5–50	5–5,000	- Different configurations available - Flexibility for different encapsulating materials - Secondary coating that offers an additional barrier to the core material	- Requires particles in a powder form to start with - Lipophilic coating (water-insoluble particles) - Cost is normally high due to processing time (2-12 h/batch) and excess of coating material - Uneven coating and exposure of the core
Emulsification	Matrix	1–100	0.2–5,000	- Carrier for hydrophilic and hydrophobic compounds - Easy to perform and scale up	- Thermodynamically unstable - Requires a drying process for the production of powders
Coacervation	Reservoir	40–90	10–800	- Easy to perform - Use one (simple) or more polymers (complex) - Load is high	- Requires a drying process to generate powder - Expensive
Liposome	Various	5–50	10–1,000	- Biodegradable and biologically inert - Encapsulation of hydrophilic and hydrophobic compounds	- Instability and leakages during storage - Low encapsulation yield - Expensive - Requires a drying process

3.3.1 Methods from which a Powder is Directly Obtained

Spray drying. Spray drying is the technique most commonly used for the encapsulation of active agents in the food industry, such as polyphenols (Robert et al. 2010), oils (Carneiro, Tonon, Grosso, & Hubinger, 2013; Frascareli, Silva, Tonon, & Hubinger, 2012; Tonon, Grosso, & Hubinger, 2011), proteins (Kurozawa, Park, & Hubinger, 2009), carotenoids (Rascón, Beristain, García, & Salgado, 2011), and probiotic living cells (Fritzen-Freire et al., 2012). It is a fast and cost-effective method that can be operated continuously and significantly increase the product's shelf life (Augustin & Hemar, 2009). The atomizing process leads to the formation of a thin film on the surface of the droplets, allowing the evaporation of small molecules and retention of the large ones (Rocha, Fávaro-Trindade, & Grosso, 2012).

The size of atomized droplets generated by the spray is intimately related to the characteristics of the solution (surface tension and viscosity), equipment setup (types of nozzle, size of nozzle orifice, spray pressure, and nozzle flow rate), among others, and will ultimately determine the drying time and particle size (Li, Anton, Arpagaus, Belleteix, & Vandamme, 2010). The spray drying process results in porous particles with high concentration of active compounds, as the temperature of the droplets is often below 100 °C (Favaro-Trindade, Santana, Monterrey-Quintero, Trindade, & Netto, 2010) for a relatively short period of time (ranging from seconds to minutes depending on the equipment) (Anwar & Kunz, 2011).

The conventional carrier materials used in spray drying are gum arabic, modified or hydrolyzed starches, and gelatin. Gum arabic (or acacia gum) consists of a mixture of arabinogalactan oligosaccharides, polysaccharides, and glycoproteins (Castellani et al., 2010). Maltodextrins are produced by hydrolysis of starch. Gelatines, on the other hand, are produced by hydrolysis of collagen and basically consist of a mixture of single- and multi-stranded polypeptides. These polymers will release the encapsulated compounds once in contact with water and the rate will depend on the porosity of the encapsulate (Sansone et al., 2011; Sun-Waterhouse, Wadhwa, & Waterhouse, 2013). The use of more hydrophobic and/or crosslinked proteins and polymers can promote a gradual release in aqueous solutions (Ortiz et al., 2009).

Although widely used, spray drying has some disadvantages. Some authors argue that this process is an immobilization and not a true encapsulation technology, as some of the bioactive compounds may be exposed on the surface (Fäldt & Bergenståhl, 1996; Minemoto, Hakamata, Adachi, & Matsuno, 2002; Rodea-González et al., 2012; Soottitantawat et al., 2005; Tonon et al. 2011). Jafari, He, and Bhandari (2007) showed that the presence of fish oil on the surface of spray-dried powder was related to the size of the particles, which would increase their susceptibility to degradation. This can be a problem for probiotics, for example, that would leak from the encapsulate into the product, affecting their viability. Another issue for probiotics is the high temperatures used for drying. In addition, the heat used in the process can cause micro-cracks on the surface of the particles that can negatively impact the stability of the encapsulated bioactive (Jones et al., 2013).

Spray cooling (or spray chilling). Although spray cooling is not often used in the food industry, it has great potential to be applied for the encapsulation of bioactive components. This technique uses a fat-based coating to encapsulate compounds dissolved in the coating solution, in an aqueous emulsion or as dry particles. It consists of the atomization of the solution in a chilled chamber (temperature is kept below the melting point of the lipid by cool air or liquid nitrogen), which will solidify the lipids and produce particles. For materials that have a melting point between 45 and 122 °C, the chamber is kept at room temperature, whereas those with lower melting points (32–42 °C) require a cooled chamber (Schrooyen, van der Meer, & De Kruif, 2001). The setup resembles that of spray drying, and the processes differ by the temperature of the drying chamber. Spray cooling does not involve the evaporation of water and the particles are less hygroscopic (Pedroso, Thomazini, Heinemann, & Favaro-Trindade, 2012). Similar to spray drying, spray cooling is a low-cost process that can be operated continuously and scaled up and could be used for the encapsulation of bioactive components such as vitamins, proteins, and probiotic cells. Examples of spray-cooled encapsulates include the studies done by Pedroso, Dogenski, Thomazini, Heinemann, and Favaro-Trindade (2014) and Pedroso et al. (2012), where the encapsulation of probiotic bacteria using palm fat and cocoa butter was reported. Here, the spray cooling process did not affect the cell viability and the

stability was improved when stored under freezing temperatures. There are, however, potential issues associated with spray cooling which include the expulsion of the bioactive during storage and incompatibility of the encapsulate with the food product in which it is added.

Freeze drying. This method is useful for heat-sensitive compounds that would degrade under the temperatures used for spray drying. If the active agent is dissolved in water, freeze drying can be used for the formation of an amorphous powder. The sample is initially frozen, followed by drying by direct sublimation at low pressure and temperature, and ground to the appropriate size. Freeze drying process can be performed alone or as an additional treatment to dry the encapsulates produced by other methods, such as emulsification and liposome encapsulation (Ezhilarasi, Indrani, Jenaa, & Anandharamakrishnana, 2014). The freeze drying technique has some disadvantages in relation to the previous methods discussed, such as high energy use, long processing time (> 20 h), and formation of porous structures that can affect the stability of the active agent. Because of these pores, freeze drying is not considered a true encapsulation process as the active ingredient is exposed to the atmosphere. In terms of price, it has been reported that freeze drying costs 30 to 50 times more than spray drying. However, freeze-dried encapsulates can exhibit improved properties, as in the study by Celli, Dibazar, Ghanem, and Brooks (2015), where maltodextrins with different dextrose equivalents (DE) were used in the preparation of blueberry extract encapsulated powder by freeze drying. Here, the encapsulates were shown to effectively retard the degradation of anthocyanins during accelerated shelf-life studies.

3.3.2 Methods that Require Additional Step for the Production of Powder

Fluid bed coating. Fluid bed or spray coating involves the application of a coating on powder particles that are typically suspended by an upward air stream. The requirements for the coating solution (which is normally a lipid) include that the viscosity should be sufficient to allow pumping and atomization, and also thermally stable, as heating is often required to avoid unwanted solidification of the coating material during pumping.

Depending on the size of the particulates and the end application of the powder, 5 to 50 % of coating can be used; however, increasing the concentration of aqueous-based coating will complicate the removal of water. It is also possible to apply a secondary coating in encapsulates prepared by other methods, such as the coating of spray-dried encapsulates containing β-carotene with hydroxypropyl cellulose (Coronel-Aguilera & Martín-González, 2015).

Emulsification. Emulsions are kinetically stable and often produced under high shear. By their nature, emulsions can be used as a vehicle for hydrophilic and hydrophobic compounds. Their stability should be guaranteed until the time of consumption for a food product, which requires the adequate selection of process and materials. A drying technique, such as spray or freeze drying, can be introduced after an oil-in-water emulsification process to generate a powder of the mixture (Tonon et al., 2011). This powder could then be used in the formulation of reconstituted beverages and other products.

Methods Based on Ionotropic Gelation. The entrapment of an active agent in gel beads, such as those prepared with alginate, can be prepared by extrusion or emulsification methods. In the extrusion method, droplets of sodium alginate solution containing the bioactive component are dropped by a syringe needle or nozzle into a bath consisting of calcium chloride for gelation. The size of particles formed varies (0.2–5 mm of diameter) depending on the dropping tool and viscoelasticity of the solution. The emulsion method, on the other hand, can be conducted in different ways, such as adding calcium chloride to an emulsion consisting of alginate aqueous solution, active agent, and vegetable oil. This last method can generate particles ranging from 10 μm to 1 mm of diameter after drying and is easier to scale up than the first technique. Both methods have been successfully applied for different bioactive components (polyphenols, probiotic cells, etc.) (Zou, Wang, Gan, & Ling, 2011). However, for the emulsion method, the material has to be extensively washed to eliminate residual oil from the surface.

Independent of the method used, ionotropic gelation is the principle that enables beads to be formed from a polymer using a counter-ion solution. Common wall materials

that are used include alginate and chitosan. Alginates are salts or esters of anionic polysaccharides constituted of α-L-guluronic (G) and β-D-mannuronic acids (M) arranged in blocks in a linear polymeric chain (alginic acid). Three blocks are normally found: M-M, G-G, and M-G (Aarstad, Tøndervik, Sletta, & Skjåk-Bræk, 2012), and their arrangement will affect the mechanical stability of the alginate. For example, the rigidity of the alginate chains decreases in the following order: GG > MM > MG (Goh, Heng, & Chan, 2012). Chitosan is also a linear polysaccharide. It is most often produced by the alkaline deacetylation of crustaceous chitin but can also be extracted from certain microorganisms and fungi. Chitosan consists of D-glucosamine and *N*-acetyl-D-glucosamine units randomly distributed in a polymeric chain and can be positively charged depending on the solution pH (p*K*a = 6.5). In the ionotropic gelation method, alginate is negatively charged and can interact with calcium ions. In the case of chitosan, the -NH_2 groups will be protonated under acidic conditions and able to interact with anions, such as TPP (Hu et al., 2008).

Coacervation. Coacervation is also based on the electrostatic interaction between polymers and salts. This interaction will result in a liquid-liquid phase separation into a polymer-rich (known as coacervate) and a polymer-poor phase (Kizilay, Kayitmazer, & Dubin, 2011). The encapsulation can occur via simple or complex coacervation, involving one or more polymers, respectively. In simple coacervation, a single polymer is used and separated by addition of agents (salts, alcohols) that have higher affinity to the water than the polymer (Martins, Rodrigues, Barreiro, & Rodrigues, 2009). Gum arabic (negative charge) and gelatin (positive charge) are commonly used as wall materials for complex coacervation, which starts an oil/water emulsion. In this case, gelatin and gum arabic are dissolved in the aqueous phase (pH adjusted to 4) under stirring at temperatures above 35 °C (gelation temperature of gelatin). As the system is cooled down, crosslinking occurs to produce particles and phase separation (Liu, Low, & Nickerson, 2010). Similar to emulsion, the coacervates can be dried to produce a powder.

The encapsulation efficiency and integrity of coacervates are influenced by the type of polymer used (mainly its molecular weight), polymer/active agent ratio, temperature, and processing time (Hao et al., 2012). Using ethyl cellulose as the coating

material, Zheng et al. (2011) reported the microencapsulation of bayberry polyphenols in particles ranging from 10 to 97 μm. The process was effective in preventing the degradation of polyphenols and, consequently, maintaining the antioxidant activity during storage. In simulated gastric (pH 2–6) and intestinal (pH 8) fluids, the release rate of polyphenols was 2.56–15.14 % and up to 87.37 %, respectively (Zheng et al., 2011). The process is still expensive compared to other techniques previously described and is often used for the development of high-value products.

Liposome. Liposomes are artificial vesicles produced with natural phospholipids (lecithin) and cholesterol as wall materials. They are biodegradable, biologically inert, and with limited toxicity and immunogenicity. Liposomes are formed when the lipid is dispersed in water under high shear. The particle size varies from 30 nm to a few microns, although the formation of aggregates has been reported during storage (van Rooijen & van Nieuwmegen, 1980).

Liposomes enable the encapsulation of virtually any compound, and its location in the vesicle will depend on solubility/lipophilicity (Coimbra et al., 2011). Liposomes have limited applications in the food industry, mainly due to their instability and leakages of the active agent during storage, low encapsulation yield, and the high costs associated with the raw materials (Karadag et al., 2013). Spray drying the liposomes has normally yielded inactive powders as the drying and rehydration processes generally degrade the components. Karadag et al. (2013) showed that coating the liposomes with chitosan prior to spray drying (with or without maltodextrin) prevented their degradation, which would enable a broader application of these encapsulates.

3.4 BIOACTIVE COMPONENTS – ENCAPSULATION AND APPLICATION IN FOOD MATRICES

Different bioactive components have been encapsulated, and the most commonly used are proteins, lipids, carbohydrates, and probiotic cells. Their physicochemical characteristics differ greatly and demonstrate the need for a tailored approach when developing encapsulated powders. Although some carbohydrates, such as the dietary

fibres (soluble non-digestible carbohydrates), are associated with biological benefits (Wannamethee, Whincup, Thomas, & Sattar, 2009), they will be considered in this review only for their role as encapsulating wall materials. In this section, we give an overview of the encapsulation of proteins, lipids, polyphenols, and probiotic living cells into powders.

Proteins. Several proteins and peptides must reach their site of action in a form where they are able to exert their biological functions, namely acting as growth factors (a review on milk-derived peptide growth factors is provided by Playford, Macdonald, & Johnson, 2000), antihypertensive agents (Contreras, Carrón, Montero, Ramos, & Recio, 2009), antioxidants (Je, Lee, Lee, & Ahn, 2009), immune regulatory factors (Horiguchi, Horiguchi, & Suzuki, 2005), and modulators of food intake (Senin et al., 2013). These compounds are very sensitive to the actions of enzymes and the different pHs that are found throughout the GI tract. In some cases, the hydrolysis and release of bioactive peptides in the upper portions of the GI tract is required for their biological function, such as in the case of casein peptides (Phelan, Aherne, FitzGerald, & O'Brien, 2009).

The direct application of proteins as well as the peptides and amino acids from protein hydrolysis is often limited in functional foods. This is due to the presence of small peptides comprised of hydrophobic amino acids that confer a bitter taste to the product (Hou, Li, Zhao, Zhang, & Li, 2011). Salty off-flavours can also result from the addition of alkalis or acids required to neutralize these hydrolysis products (Hernández-Ledesma, Contreras, & Recio, 2011; Leksrisompong, Miracle, & Drake, 2010). In addition, some compounds can be lost during Maillard reactions with reducing carbohydrates. Favaro-Trindade et al. (2010) reported that spray drying casein hydrolysates with gelatine and soy protein isolate attenuated the bitter taste and hygroscopicity associated with the core material, and the resulting powder was compatible with oil and aqueous formulations. Rocha, Trindade, Netto, and Favaro-Trindade (2009) also reported a reduction of bitterness by using two types of maltodextrins with DE of 10 and 20 as the polymeric matrix. The application of this powder in protein bars received better scores in the sensory panel than those prepared with free hydrolysate. In the work by Contreras et al. (2011), spray-dried powders prepared with casein peptides and lactose were incorporated

in pasteurized liquid yoghurt, where it was demonstrated that these bioactives were stable for 1 month when stored at 4 °C.

Lipids. Several compounds are included in this group, such as fatty acids, phospholipids, carotenoids, phytosterols, and oil-soluble vitamins. Encapsulation of lipids has contributed to a broader application of these compounds by the food industry, circumventing previous limitations such as low water solubility and high susceptibility to oxidation due to the presence of polyunsaturated chains (Verardo et al., 2009). For the sake of this review, carotenoids and omega (ω)-3 fatty acids will be used to exemplify the encapsulation and application of lipids by the food industry.

Carotenoids are a diverse class of compounds that have been associated with several health benefits. For instance, lutein and zeaxanthin are found in the macula and lens of the human eye and associated with macular degeneration and cataracts (Ma et al., 2012). Reports suggest that the application of carotenoids in food products leads to higher bioavailability than when they are consumed in their natural sources (Ghavami, Coward, & Bluck, 2012), although it would depend on the ability of the body to obtain these compounds from the food matrix. However, the use of extracted carotenoids in their pure form in food products is limited due to factors such as high melting point (Sy et al., 2012), low solubility in water, flavour, and stability issues. An example of this is the presence of iron, a common prooxidant widely found in food products, that can lead to the degradation of carotenoids (Boon, McClements, Weiss, & Decker, 2009). These compounds can also undergo isomerization reactions due to processing, such as heating (Chen & Tang, 1998) and drying (Goula, Adamopoulos, Chatzitakis, & Nikas, 2006), or oxidative degradation (Mordi et al., 1993). In a recent study, Paz, Martín, and Cocero (2013) developed a water-soluble β-carotene powder for possible application in reconstituted beverages by using a nanoemulsion precipitation stabilized with modified starch, followed by spray drying.

Omega-3 fatty acids also have several health-promoting benefits that include influencing cell membrane fluidity, gene expression, etc. The most common ω-3 fatty acids are α-linolenic (ALA), eicosapentaenoic (EPA), and docosahexaenoic acids (DHA) (Egert, Kannenberg, Somoza, Erbersdobler, & Wahrburg, 2009). EPA and DHA are

considered the compounds with highest bioactivity in this group. One of the main sources of these fatty acids is fish oil; however, a limitation of using fish oil in food products is the high susceptibility to oxidation, which leads to the formation of degradation products that have an unpleasant smell and taste (Fournier et al., 2006). Comparing fish oil soft-gel capsules and spray-dried complex coacervates containing ω-3 fish oil added to a milkshake, Barrow, Nolan, and Holub (2009) showed that the bioavailability did not differ between the treatments in human volunteers. Both treatments reduced the levels of blood triglycerides after 21 days of intake. Iafelice et al. (2008) reported the incorporation of encapsulated marine oil powder (minimum 30 % EPA and DHA in a cornstarch matrix coated with fish gelatine and sucrose) in spaghetti and showed that the processing conditions during pasta preparation and cooking did not significantly affect the content of fatty acids. No significant differences were observed between the pasta enriched with the powder and the control in the sensory panel.

Polyphenols. At present, no health claims are currently approved by the US FDA or Health Canada for polyphenols. In Europe, the Commission Regulation No. 432/2012 approved the claim "olive oil polyphenols contribute to the protection of blood lipids from oxidative stress", for those olive oils containing at least 5 mg of hydroxytyrosol and its derivatives per 20 g of oil (European Commission, 2012). In Japan, guava tea polyphenols are an approved Food for Specified Health Uses (FOSHU) for foods related to blood sugar levels.

However, research has shown that the daily consumption of fruits is positively associated with health benefits and reduced incidence of degenerative diseases, and polyphenols are considered to be the main contributors resulting in these benefits (Oyebode et al., 2014). The term polyphenol encompasses a large class of secondary metabolites that include compounds such as stilbenes (e.g. resveratrol), flavonols (e.g. quercetin), and anthocyanidins (e.g. cyanidin). Encapsulation technology can greatly increase the application of these compounds in functional foods, as properties such as stability, solubility, and bioavailability can be modified and made more suitable for this use. An example is given by Ezhilarasi et al. (2014), where *Garcinia cowa* fruit extract was spray dried with maltodextrin for incorporation into bread. These breads exhibited

higher phytochemical content and organoleptic characteristics than a control prepared with aqueous extract. Pillai, Prabhasankar, Jena, and Anandharamakrishnan (2012) also showed that the spray-dried extract of these fruits (with whey protein) was positively associated with better sensory characteristics and antioxidant activity of pasta in comparison to a control. In addition, Burin, Rossa, Ferreira-Lima, Hillmann, and Boirdignon-Luiz (2011) reported the encapsulation of anthocyanins from Cabernet Sauvignon grapes by spray drying using different wall materials and further application in an isotonic soft drink model.

Probiotic living cells. These bacteria and yeasts positively impact the host's health, contributing to the microbial balance and conferring health benefits when administered in adequate amounts (FAO/WHO, 2002). To be considered probiotic, the microorganisms must be viable. Although these living cells can be incorporated in a wide range of products, limitations include their low survival rate and degradation of bioactive glycoproteins, which is influenced by several factors, including pH (for the product and *in vivo*), oxygen, and lack of nitrogen source (López-Rubio, Sanchez, Sanz, & Lagaron, 2009). The goal of encapsulation is to protect the cells against adverse conditions during processing, storage, and those found throughout the GI tract, ensuring that the microorganisms are metabolically active once they reach their site of action. Encapsulated powders containing probiotic cells and prepared by spray and freeze drying have been successfully applied in foods such as Crescenza (Gobbetti, Corsetti, Smacchi, Zocchetti, & De Angelis, 1998) and cheddar cheese (Gardiner et al., 2002), yoghurt (Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-Ramírez, & Vernon-Carter, 2010), and chocolate (Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010).

3.5 PROPERTIES OF THE ENCAPSULATED POWDER

Food powders in general are easier and cheaper to store and transport than other forms due to the low water content (Cuq, Rondet, & Abecassis, 2011), which also helps to maintain the product stable for longer periods of time (Descamps, Palzer, Roos, & Fitzpatrick, 2013; Fitzpatrick, 2007). Similar to other powders, the physicochemical

properties of encapsulated powders can be divided into primary or fundamental properties (e.g. density, shape, etc.) and secondary or functional properties (e.g. settling velocity, rehydration rate, etc.). This distinction is based on the former group dictating the latter properties, and both groups of properties are used to assess the behaviour of the powder and should be considered for their application in functional foods and beverages (Cuq et al., 2011; Kim, Chen, & Pearce, 2009). However, the information obtained from fundamental properties is not often sufficient to predict the technological behaviour of the powder under processing conditions (Cuq et al., 2011).

Fitzpatrick and Ahrné (2005) suggested that it might be useful to first investigate and evaluate how the powder will be applied prior to its development and then work backwards to determine the best method of production. The objective of this section is to discuss the implications of the encapsulated powder characteristics on the development, production, and handling of functional foods and beverages, and it is not intended to present methods for their measurement. Table 3.2 summarizes some studies that investigated the application of powdered encapsulates in food products.

Table 3.2 Examples of encapsulated bioactive components, their application in food products, and properties assessed

Bioactive	Encapsulation technique	Material for encapsulation	Food product	Powder properties reported	Quality measure reported	Reference
Casein hydrolysate	Spray drying	Maltodextrin (DE 10 and 20)	Protein bar	Dissolution Hygroscopicity Moisture content Morphology Particle size Sensory evaluation T_g Water activity	Moisture content Sensory evaluation Water activity	Rocha et al., 2009
Casein hydrolysate	Spray or freeze drying	Lactose	Yoghurt	Protein content Moisture content Mineral content Sensory evaluation	Sensory evaluation Stability over time	Contreras et al., 2011
ω-3 fatty acids	Coacervation followed by spray drying	Gelatin	Milkshake	-	-	Barrow et al., 2009
ω-3 fatty acids	n.s.	Cornstarch, gelatin, sucrose	Spaghetti	-	Fatty acid composition Lipid content Sensory evaluation	Iafelice et al., 2008

Bioactive	Encapsulation technique	Material for encapsulation	Food product	Powder properties reported	Quality measure reported	Reference
Fish oil	n.s.	n.s.	Fermented sausage	-	Firmness Lipid oxidation Moisture content pH Protein content Sensory evaluation Total fat and fatty acid composition	Josquin, Linssen, & Houben, 2012
Probiotic cells (*Bifidobacterium bifidum* 15696)	Ionotropic gelation followed by freeze drying	κ-carrageenan	Cheddar cheese	Morphology	Ash Metabolic activity of microorganisms Microbiological profile Moisture content Sensory evaluation Total protein, fat, and salt contents	Dinakar & Mistry, 1994
Probiotic cells (*Lactobacillus paracasei* NFBC 338)	Spray drying	Skim milk	Cheddar cheese	Bulk density Moisture content Viability Whey protein nitrogen index	pH Moisture content Salt, protein, and content Sensory evaluation Viability	Gardiner et al., 2002

Bioactive	Encapsulation technique	Material for encapsulation	Food product	Powder properties reported	Quality measure reported	Reference
Probiotic cells (*B. breve* R070 and *B. longum* R023)	Freeze drying followed by emulsification and spray drying	Whey protein and milk fat	Yoghurt	Moisture content Morphology Relative humidity	pH Viability over time	Picot & Lacroix, 2004
Probiotic cells (*L. acidophyllus* CSCC2400 and CSCC2409) and prebiotic oligosaccharides (inulin, oligofructose, high amylose corn starch)	Ionotropic gelation	Alginate, chitosan or poly-L-lysine	Yoghurt	Particle size Viability	pH Viability over time	Iyer & Kailasapathy, 2005
Probiotic cells (*L. reuteri* ATCC55730)	Ionotropic gelation	Alginate	Dry fermented sausage	Viability	pH Sensory evaluation Viability over time Water activity	Muthukumaras amy & Holley, 2006
Probiotic cells (*L. helveticus* CNCM I-1722 and *B. longum* CNCM I-3470)	Freeze drying	n.s.	Chocolate	-	Capacity to colonize colon (simulated long-term consumption) Effect on microbiota Probiotic content Viability	Possemiers et al., 2010

Bioactive	Encapsulation technique	Material for encapsulation	Food product	Powder properties reported	Quality measure reported	Reference
Garciania cowa fruit extract	Spray drying	Whey protein and maltodextrin	Bread	Moisture content Morphology	Crumb firmness and color Hydroxycitric acid content Loaf volume Moisture and ash content Sensory evaluation	Ezhilarasi et al., 2014
Garciania cowa fruit extract	Spray drying	Whey protein	Pasta	Antioxidant activity Moisture content Morphology	Antioxidant activity Cooking quality Sensory evaluation	Pillai et al., 2012
Anthocyanins	Spray drying	Maltodextrin (DE 19) with or without arabic gum or γ-ciclodextrin	Isotonic soft drink	Color Morphology	Anthocyanin stability under light	Burin et al., 2011

The fundamental properties of the powder, such as particle size and morphology, are intrinsic characteristics that can be assessed directly after their preparation and do not depend on environmental conditions (Cuq et al., 2011). Particle size and its distribution are the main contributors to the bulk density, compressibility, and flowability of the powder (Zhao, Yang, Gai, & Yang, 2009). The flowability is also determined by the particle surface. This property is more relevant during processing than for the consumer, because it determines how the powder will be transported, mixed, and processed into a food product. In the same way, bulk density will determine the appropriate storage, processing, and packaging conditions for the powder.

Particle size distribution and morphology can also be used as an indication of stickiness and caking. These are often observed in powders under certain conditions and are related to the glass transition temperature (T_g). Encapsulated powders might contain components in amorphous glassy state, such as those produced by spray and freeze drying. These components are thermodynamically unstable and can become sticky when the temperature is raised above their T_g (by approximately 10–20 °C), due to an increase of surface energy of the particulates that will enable interactions with other molecules and adhesion to solid surfaces (Fitzpatrick, 2007). Powders in the amorphous state are also prone to caking during storage, which will result in undesirable reactions and potential losses of the encapsulated bioactive compounds and quality (Telis & Martínez-Navarrete, 2009). Caking can result from temperatures above the T_g, amount of amorphous material, and absorption of moisture, which forms a liquid film on the surface of the particles (Goula & Adamopoulos, 2010) that increase their cohesiveness (Fitzpatrick et al., 2007). Fitzpatrick (2007) also noted that cohesiveness could result from the surface composition. Comparing milk powders with different milk fat contents (1, 26, and 73 %), this author observed that the higher fat concentration was related to more cohesiveness since these compounds tend to migrate to the surface during drying.

Segregation of the powder becomes important when working with dry mixtures containing the encapsulated bioactive and occurs mainly due to differences in particle size and density. The appropriate selection of ingredients that share similar physical characteristics as those of the encapsulated bioactive is recommended to avoid

segregation of these powders and guarantee the homogeneity and concentration of the functional ingredient in the final product (Fitzpatrick & Ahrné, 2005).

If a mixture of powders is used for the preparation of reconstituted drinks, for example, some characteristics should be considered, namely wettability, dispersibility, and solubility, in addition to the properties previously mentioned. Wettability is related to the capacity of the powder to absorb water on its surface and is the initial stage of the reconstitution process. It is affected by particle size and the presence of lipids on the surface. Dispersibility is related to the distribution of the particles in the liquid, whereas solubility refers to the extent and rate of dissolution of these particles. It is also possible that the different powders in the mixture will exchange moisture if their water activities are different (Hartmann & Palzer, 2011).

Bakery products offer additional challenges for the application of encapsulated bioactive components as the process involves high shear and high temperatures. Excessive attrition generated during processing can have deleterious effects on the integrity of the particulates (Perfetti, Aubert, Wildeboer, & Meesters, 2011) and, consequently, the stability and release of the bioactive ingredient. For this reason, the mechanical strength of the particles should also be considered, such as abrasiveness, hardness, and friability.

High shear forces are also observed during the manufacture of chocolate in the milling and conching processes. These processes involve a frictional mixture consisting of ingredients such as cocoa products, sugar, milk powder, etc. This stage aims, among other things, to reduce the particle size (< 30 μm) (Beckett, 2008). For this reason, if an encapsulate were to be incorporated in chocolate, it should be added in the final stages during mixture when no friction is needed. Here, dispersibility is also an important characteristic, as the powder should be distributed throughout the chocolate mass and not form aggregates.

Josquin et al. (2012) evaluated the incorporation of encapsulated, pure, and pre-emulsified fish oil in the preparation and quality of Dutch-style fermented sausages, with partial substitution of pork fat. The use of encapsulated fish oil powder resulted in lower lipid oxidation in comparison to the other treatments, measured by the concentration of hexanal and proponal in the package headspace. However, it is not clear if this

observation is due to the encapsulation or presence of other compounds in the powder, such as ascorbic acid. When assessed by their sensory characteristics, sausages containing encapsulated oil powder were firmer than the ones produced with pure oil and the sensory panel identified these differences. Another improvement using the encapsulated oil powder was the reduced feeling of an oily product that was perceived in sausages with pure oil (Josquin et al., 2012).

With the exception of beverages and reconstituted powder mixtures, the bioaccessibility is a characteristic that should be assessed after the incorporation of the encapsulated powder in a functional food. This term refers to the amount of bioactive ingested that is available for absorption after digestion and measures the efficiency of the body in "extracting" these compounds from the food matrices (Pool, Mendoza, Xiao, & McClements, 2013). Although the encapsulates are often designed to increase the availability of certain compounds, the food matrix in which they are incorporated can restrict their extraction and utilization. For instance, adsorption and association with other compounds would have a marked effect on the anthocyanin concentration. The weak association of these compounds with other natural substances (amino acids, alkaloids, other phenolics, etc.) has been widely described in the literature (Di Meo, Sancho Garcia, Dangles, & Trouillas, 2012; Ngo & Zhao, 2009). This is a unique feature of anthocyanins and serves to protect the flavylium cation from nucleophilic attack and, consequently, stabilize the structure (Di Meo et al., 2012). An example is given by Aura et al. (2005), who showed that the recovery of anthocyanins from spiked faeces was incomplete, indicating that these compounds are binding to the faecal matrix (mainly constituted of undigested material).

3.6 CONCLUSIONS

A considerable body of research is available on the encapsulation of bioactive ingredients, and this review only presented some examples from the literature. Similar to the pharmaceutical field, there is still a large gap between the development of technologies and encapsulates and their commercial applications. A major limitation is related to the scale up of processes. Taking ionotropic gelation by extrusion as an

example, it is common for research studies to perform this technique using a bench-top system consisting of a syringe and needle, which is not often practical for the production of large volumes. The costs for new technologies can also be prohibitive for a broader use by the industry. In the case of probiotic cells, a major challenge is in producing encapsulates of a sufficiently small size to limit the impact on sensory and textural characteristics that could negatively impact the consumer's acceptance while keeping the cells viable or the molecules bioactive.

There is still a significant knowledge gap in the current research regarding the application of encapsulates in food products. As discussed in the previous section, an encapsulate powder must have particular physicochemical properties to ensure that it is suitable for incorporation into particular food products, and this will mainly depend on the type of food and the processes involved in its production. Moreover, the encapsulates can also impact the end product, potentially influencing its sensory attributes. It is evident that the studies vary in the level of detail used to characterize the powders, with some reporting several parameters and others neglecting to report on the physicochemical properties at all. In addition, most studies include sensory evaluation for assessing the quality of the end food product, as well as additional parameters such as viability for probiotics and antioxidant activity for fruit extracts. Another important consideration that is not included in any of these studies and that is commonly overlooked is the fate of the encapsulated bioactive compounds after consumption. This is arguably the most significant factor and should be used to determine the quality of the overall food product, as these encapsulated powders have no utility as functional food components if they are not bioaccessible or fail to deliver the bioactive component to the correct site *in vivo* to facilitate biological activity.

Another gap is in the regulations of functional foods and encapsulated bioactive components that are not clear in some cases and vary widely between countries. For instance, the health claims that have been permitted in Canada are far fewer than in Japan, where there are over 500 functional foods approved. For a product to be considered a functional food, its efficacy and safety should be thoroughly evaluated and demonstrated. This requires assessment of each different functional food product, which is a significant undertaking, but is necessary as the efficacy of a functional food product

will be dependent on the type of matrix material used for the encapsulated particles, of which there are many possibilities.

These limitations should be considered as the focus for future work. Further research should involve the development or improvement of technology for practical applications in large-scale processes. It should also aim at determining and demonstrating the potential interactions and efficacy of encapsulated bioactive compounds with different food products, which is currently one of the requirements to make health claims. In addition, research institutions should work collaboratively with industry and regulatory agencies to improve current regulations.

Chapter 4 A THEORETICAL PHYSIOLOGICALLY-BASED PHARMACOKINETIC APPROACH FOR MODELLING THE FATE OF ANTHOCYANINS *IN VIVO*

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4.1 ABSTRACT

Recent studies on the pharmacokinetics of anthocyanins (ACNs) and their metabolites have uncovered evidence for hitherto unknown physiological effects affecting the fate of these compounds *in vivo*. In particular, it has been shown that the stomach, in addition to the small intestine, has an important role in absorption. Most studies still use a non-compartmental or one-compartmental approach to determine the pharmacokinetic parameters of ACNs, which does not represent the anatomical and physiological conditions that a compound is subject to in the organism. Thus, the objective of this study was to review the current knowledge of the different processes involved in the metabolism of ACNs once ingested and, based on this information, propose a theoretical physiologically-based, multi-compartmental pharmacokinetic (PBPK) model to describe their fate *in vivo*. This is the first study that reports a PBPK model for ACNs; the model provides a more physiologically representative approach for ACN metabolism, which could be used as a basis for experimental designs and interspecies scale-up (i.e. to predict the pharmacokinetics in humans based on results from preclinical trials).

4.2 INTRODUCTION

The pharmacokinetics (PK) of exogenous compounds refer to their absorption, distribution, metabolism, and excretion (ADME) (Petzinger & Geyer, 2006). For some time, the PK of anthocyanins (ACNs) was unknown and thought to be nearly inexistent as their absorption was considered limited (Nemeth et al., 2003). Although the whole process is not completely understood, researchers have indicated that existing studies have underestimated the amount of ACNs absorbed (Fernandes, de Freitas, Reis, & Mateus, 2012) as recent reports have highlighted higher than expected bioavailability rates and longer permanence time of ACN-related metabolites in the circulatory system (Czank et al., 2013; Kalt, Liu, McDonald, Vinqvist-Tymchuk, & Fillmore, 2014; Kay et al., 2013).

A limitation of the methods used for the identification and quantification of ACNs and their metabolites in biological fluids is the indirect assessment of the flavylium cation, which is the ACN form that predominates at pH < 2. As a consequence, structures that are not able to regenerate this ion by reacidification of the sample due to *in vivo* metabolism and/or modifications in the chemical structure will not be detected (Fernandes et al., 2012). In addition, some studies have reported a lower than expected recovery of parent ACNs from plasma and urine after ingestion, which could not be representative of the compounds found *in vivo* after metabolism and excretion (He, Magnuson, & Giusti, 2005). Another issue is the low concentration of some metabolites that would be present, which would be near the limit of the detection of the methods and equipment used (Czank et al., 2013; Kay, Mazza, Holub, & Wang, 2004), potential degradation of these compounds during sample preparation (Felgines et al., 2003; He et al., 2005), and binding of the compounds and metabolites to proteins in the bloodstream (Al Bittar, Mora, Loonis, & Dangles, 2014; Tang, Zuo, & Shu, 2014).

Current studies often use a non-compartmental or one-compartmental approach to assess bioavailability of ACNs. Although simple, these methods do not appropriately represent the anatomical, physiological (e.g. tissue volumes and blood flow), and biochemical conditions (e.g. metabolic rate constants) that a particular compound encounters in the organism, in association with their physicochemical characteristics (e.g.

partition coefficient) (Pastino & Conolly, 2000). For this reason, a theoretical physiologically-based pharmacokinetic (PBPK) model is proposed to better describe the bioavailability of ACNs. Here, a review of the current literature related to the PK of ACNs is presented. This information is then used to develop a PBPK model that accounts for their passage through different compartments in the body, taking into consideration the different variables that impact their PK. This model will ultimately provide insights on the absorption, distribution, metabolism, and excretion of ACNs and their metabolites, and could contribute to the experimental design of *in vitro* and *in vivo* studies taking into account the physiologically relevant concentration of circulating compounds. To the best of our knowledge, this is the first time a PBPK model is used to describe the fate of ACNs *in vivo*.

4.3 PHARMACOKINETICS OF ANTHOCYANINS

After ingestion, exogenous compounds are generally absorbed in the gastrointestinal (GI) tract, distributed by the systemic circulation, metabolized, and finally excreted. In the case of ACNs, in addition to absorption in the small intestine, a considerable body of evidence has demonstrated that the stomach could also have a role (Passamonti, Vrhovsek, Vanzo, & Mattivi, 2003). It is also worth noting that ACNs can be found in different structures, which results in varied PK parameters and pharmacological effects (Pojer, Mattivi, Johnson, & Stockley, 2013). Tables 4.1 and 4.2 summarize some of the PK parameters for ACNs described in the literature for animal models and in humans, respectively. The following sections review the literature available for the PK of ACNs, where the absorption, distribution, metabolism, and excretion are described.

4.3.1 Absorption

Oral absorption rates differ greatly among polyphenols and can be influenced by chemical (e.g. pH), biological (e.g. intestinal microbiota and enzymes), and physiological conditions (e.g. barriers, presence of other nutrients, disorders and/or pathologies) along

the GI tract (Ugalde et al., 2009), in addition to the chemical structure and the matrix where the compounds are found (Charron et al., 2009). For example, Charron et al. (2009) showed that nonacylated ACNs have higher bioavailability when compared to acylated ACNs. When comparing different food matrices, the total amount of ACNs absorbed was similar when ingested as carrot juice and whole carrots; however, the absorption of these compounds from the juice sample was faster since it was not subject to the same digestive time required for the whole carrot.

Research has suggested that the stomach could have a role in the absorption of ACNs (Cai et al., 2011; Felgines et al., 2007), which could justify the rapid detection of these compounds in plasma and where the pH conditions favor their chemical stability (Woodward, Needs, & Kay, 2011). At physiological gastric pH, ACNs are likely to be found in the flavylium cation form (depending on the p*K*a of the compound) (Fernandes et al., 2012), where absorption by passive diffusion is likely to be limited (Lipinski, Lombardo, Dominy, & Feeney, 1997). However, it has been suggested that ACN absorption in the stomach could occur via bilitranslocase, an organic anion carrier (Passamonti et al., 2003, 2005a), where increasing the concentration of ACNs would ultimately result in the saturation of this carrier and reduction of their absorption (Fernandes et al., 2012; Kurilich, Clevidence, Britz, Simon, & Novotny, 2005; Talavéra et al., 2003).

According to Vanzo, Vrhovsek, Tramer, Mattivi, and Passamonti (2011), the involvement of bilitranslocase in the absorption process could justify the rapid uptake of cyanidin 3-glucoside after intravenous administration in Wistar rats. In their study, they detected peonidin 3-glucoside (a methylated derivative of cyanidin 3-glucoside) within 15 s after the administration of cyanidin 3-glucoside and attributed this to the rapid cellular uptake of the parent ACNs, methylation, and transport of the metabolite to the plasma. These results are supported by the concomitant (and rapid) reduction of the cyanidin 3-glucoside concentration in plasma. It is believed that bilitranslocase could mediate the influx of cyanidin 3-glucoside and efflux of the metabolites to the circulatory system (Vanzo et al., 2011).

Table 4.1 PK parameters for ACNs from studies performed in animal models

Animal	ACN source	Dose[a]	C_{max}[b]	t_{max} (h)[c]	AUC[d]	Urinary excretion (%)[e]	Reference
Rat	Bilberry (extract)	400 mg	2–3 µg/mL	0.25			Morazzoni, Livio, Scilingo, & Malandrino, 1991
	Black currant (extract)	489 mg delphinidin 3-rutinoside	0.36 mg/L	2	0.81 mg h/L		Matsumoto et al., 2001
		476 mg cyanidin 3-rutinoside	0.51 mg/L	0.5	1.51 mg h/L		
		359 mg cyanidin 3-glucoside	0.38 mg/L	0.5	0.68 mg h/L		
	Black currant (extract)	100 mg delphinidin 3-glucopyranoside	0.19 mg/L	0.25			Ichiyanagi et al., 2004
	Elderberry/black currant (extract)	320 mg cyanidin 3-glucoside 40 mg cyanidin 3,5-diglucoside	1.56 mg/L 0.19 mg/L	0.25	1.62 mg h/L		Miyazawa, Nakagawa, Kudo, Muraishi, & Someya, 1999
	Purple corn (extract)	404 mg cyanidin 3-glucoside	0.14 mg/L	0.5			Tsuda, Horio, & Osawa, 1999
Rabbit	Black currant (extract)	117 mg	780 µg/L	0.5	6.6 µg/L	0.035 (4 h)	Nielsen, Dragsted, Ravn-Haren, Freese, & Rasmussen, 2003
		164 mg	100 µg/L		0.76 µg/L	0.009 (4 h)	
		53 mg (delphinidin glycosides)	450 µg/L		1.8 µg/L	0.023 (4 h)	
Pig	Marionberry (powder)	74.2 mg	16 µg/L (C3G) 9.2 µg/L (C3Rut)	1	29.1 µg/L 24.7 µg/L	0.088 (24 h)	Wu, Pittman, & Prior, 2004

[a] amount per kg of body weight (BW). Total amount of ACNs (if not specified)

[b] maximal plasma concentration

[c] time to reach maximum plasma concentration (C_{max})

[d] area under the curve

[e] % of initial dose. The numbers in parenthesis indicate the time after ingestion when the assessment was made

Abbreviations: C3G – cyanidin 3-glucoside; C3Rut – cyanidin 3-rutinoside

Table 4.2 PK parameters for ACNs estimated in humans

ACN source	Dose[a]	C_{max}[b]	t_{max} (h)[c]	AUC[d]	Urinary excretion (%)[e]	Reference
Bilberry/lingonberry (puree)	650 mg	62 µg/L	1.5			Nurmi et al., 2009
Blackberry (fruit)	431 mg				0.16 (24 h)	Felgines et al., 2005
Blackcurrant (extract)	111 mg delphinidin 3-rutinoside	45 µg/L	1.75	176 µg h/L	0.11 (8 h)	Matsumoto et al., 2001
	82 mg cyanidin 3-rutinoside	27 µg/L	1.50	99.8 µg h/L		
	32 mg delphinidin 3-glucoside	11 µg/L	1.50	32 µg h/L		
	11 mg cyanidin 3-glucoside	2.3 µg/L	1.25	4.1 µg h/L		
Blackcurrant juice (200 mL)	153 mg				0.02–0.05 (5 h)	Netzel, Strass, Janssen, Bitsch, & Bitsch, 2001
Blackcurrant (juice)	1095 mg (undiluted juice)	53 µg/L	0.75	19 µg h/L	0.072 (4 h)	Nielsen et al., 2003
	672 mg (juice)	16 µg/L	0.75	19 µg h/L	0.048 (4 h)	
	672 mg (juice+rice cake)	32 µg/L	1.5	16 µg h/L	0.045 (4 h)	
Blackcurrant (concentrate)	188.5 mg				0.064 (7 h)	McGhie, Ainge, Barnett, Cooney, & Jensen, 2003
Blood orange (juice)	71 mg cyanidin 3-glucoside	0.85 µg/L	0.5	3.1 µg h/L	1.2 (24 h)	Vitaglione et al., 2007
Blueberry (powder)	1200 mg	13.1 µg/L	4			Mazza, Kay, Cottrell, & Holub, 2002
Blueberry (extract)	439.1 mg				0.02 (7 h)	McGhie et al., 2003
Boysenberry (concentrate)	344.5 mg				0.029 (7 h)	McGhie et al., 2003

ACN source	Dose[a]	C_{max}[b]	t_{max} (h)[c]	AUC[d]	Urinary excretion (%)[e]	Reference
Chokeberry (extract)	1300 mg cyanidin 3-glycosides	265 µg/L	0.5–2			Kay et al., 2004
Chokeberry (extract)	721 mg cyanidin 3-glycosides	43.2 µg/L	2.8	169 µg h/L	0.15 (24 h)	Kay, Mazza, & Holub, 2005
Cranberry (juice)	94.47 mg	2.15 µg/L	~3	8.7 µg h/L	0.078–3.2 (4 h)	Milbury, Vita, & Blumberg, 2010
Dealcoholized red wine	58 mg malvidin 3-glucoside	0.7 µg/L	1.5	106 µg h/L	<0.03 (6 h)	Bub, Watzl, Heeb, Rechkemmer, & Briviba, 2001
Elderberry (extract)	1500 mg	100 µg/L	0.5			Cao & Prior, 1999
Elderberry (juice)	180 mg	35 µg/L	1			Murkovic, Adam, & Pfannhauser, 2000
Elderberry (juice)	500 mg				0.05 (6 h)	Murkovic, Mulleder, Adam, & Pfannhauser, 2001
Elderberry (extract)	720 mg	43.8 µg/L	1.2		0.05 (24 h)	Cao, Muccitelli, Sanchez-Moreno, & Prior, 2001
Elderberry (concentrate)	1900 mg				0.03–0.012 (6 h)	Mülleder, Murkovic, & Pfannhauser, 2002
Elderberry (extract)	720 mg	43.8 µg/L	1.2		0.05 (24 h)	Milbury, Cao, Prior, & Blumberg, 2002
Elderberry (juice)	3570 mg				0.053 (5 h)	Bitsch et al., 2004b

ACN source	Dose[a]	C_{max}[b]	t_{max} (h)[c]	AUC[d]	Urinary excretion (%)[e]	Reference
Elderberry/black currant (extract)	148 mg cyanidin 3-glucoside 13.7 mg cyanidin 3,5-diglucoside	13 µg/L	0.5			Miyazawa et al., 1999
Hibiscus (extract)	147.4 mg	3.4 µg/L	1.5	7.4 µg h/L	0.018 (7 h)	Frank et al., 2005
Red grape (juice)	117 mg malvidin 3-glucoside	1.5 µg/L	2	327 µg h/L	<0.03 (6 h)	Bub et al., 2001
Red grape (juice)	283.5 mg	100 µg/L	0.5	168.4 µg h/L	0.23 (7 h)	Frank, Netzel, Strass, Bitsch, & Bitsch, 2003
Red grape (juice)	283.5 mg	100 µg/L	0.5	168.4 µg h/L	0.23 (7 h)	Bitsch, Netzel, Frank, Strass, & Bitsch, 2004a
Red wine	218 mg				1.5–5.1 (12 h)	Lapidot, Harel, Granit, & Kanner, 1998
Red wine	68 mg malvidin 3-glucoside	0.7 µg/L	0.8	142 µg h/L	<0.03 (6 h)	Bub et al., 2001
Red wine	279.6 mg	42.9 µg/L	1.5	100 µg h/L	0.18 (7 h)	Frank et al., 2003
Red wine	279.6 mg	42.9 µg/L	1.5	100 µg h/L	0.18 (7 h)	Bitsch et al., 2004a
Strawberry (fruit)	77 mg pelargonidin 3-glucoside				1.8 (24 h)	Felgines et al., 2003

[a] amount per kg of body weight (BW). Total amount of ACNs (if not specified)

[b] maximal plasma concentration

[c] time to reach maximum plasma concentration (C_{max})

[d] area under the curve

[e] % of initial dose. The numbers in parenthesis indicate the time after ingestion when the assessment was made

ACNs are also absorbed in the small intestine. It has been suggested that polyphenol glycosides are first hydrolyzed to their aglycone forms prior to absorption, and with the exception of cyanidin and delphinidin glucosides (Nemeth et al., 2003), ACNs and other flavonoids can be hydrolyzed at the mucosal brush-border membrane by β-hydroxylase LPH (Day et al., 2000). To account for the presence of parent ACNs in blood and urine, a mechanism of absorption was proposed involving the sodium-glucose co-transporter (SGLT) carrier, similarly to other flavonoids (Cao et al., 2001; McGhie et al., 2003; Tsuda et al., 1999). However, Felgines et al. (2008) demonstrated that the intestinal absorption of ACNs in rats was not affected by concomitant administration of glucose, which could indicate that their transport is not mediated by SGLT.

In situ perfusion of ACNs in the jejunum and ileum of Wistar rats for 45 min resulted in the absorption of high amounts of ACN glycosides. The exact amount varied depending on the compound structure: between 10.7 (malvidin 3-glucoside) to 22.4 % (cyanidin 3-glucoside) (Talavéra et al., 2004). Absorption was also affected by the sugar moiety as the uptake of cyanidin 3-glucoside was significantly higher than its 3-galactoside and 3-rutinoside derivatives. However, He, Wallace, Keatley, Failla, and Giusti (2009) attributed the reduction of cyanidin 3-glucoside concentration in intestinal fluid to degradation, and not higher absorption.

4.3.2 Distribution

Andres-Lacueva et al. (2005) and Passamonti, Vrhovsek, Vanzo, and Mattivi (2005) demonstrated that ACNs can cross the blood-brain barrier in blueberry-fed rats. These compounds were identified in regions of the brain related to learning and memory after an 8-week exposure period (Andres-Lacueva et al., 2005). Intact blackcurrant ACNs also crossed the blood-aqueous and blood-retinal barriers after oral administration in rats (Matsumoto, Nakamura, Iida, Ito, & Ohguro, 2006). Using healthy weanling pigs as a model for human nutrition, Kalt et al. (2008a) investigated the effects of long-term exposure (four weeks) of these animals to blueberries (1, 2, and 4 %, w/w, added to the basal diet). Pigs were used in this study due to similarities with humans in relation to digestive absorption and cardiovascular physiology. Eleven intact ACNs were reported in

the brain cortex, liver, eye, and cerebellum tissues, whereas none were detected in the plasma and urine (Kalt et al., 2008a).

ACNs and their metabolites can bind to human serum albumin (HSA) under physiological pH conditions (Wiese, Gärtner, Rawel, Winterhalter, & Kulling, 2009), similarly to other polyphenols, which would affect their half-life in plasma and tissue distribution. Indeed, HSA is known to mediate the transport of a large variety of ligands (Varshney et al., 2010) and it has been suggested that polyphenols would bind to sites characterized by the presence of hydrophobic pockets containing positive charges due to arginine and lysine amino acid residues (Al Bittar et al., 2014). In *in vitro* conditions, Al Bittar et al. (2014) demonstrated that chalcones, an ACN metabolite, have higher affinity for HSA than their parent compounds, possibly due to their linear structure. It has also been demonstrated that increasing the number of hydroxyl substituents in ring B of the ACN backbone structure contributes to a higher binding affinity to HSA (e.g. delphidin 3-glucoside has higher affinity than cyanidin 3-glucoside) (Tang et al., 2014) (Figure 4.1). Since albumin can escape reabsorption in the body through the kidneys, it could contribute to the elimination of ACNs via urine.

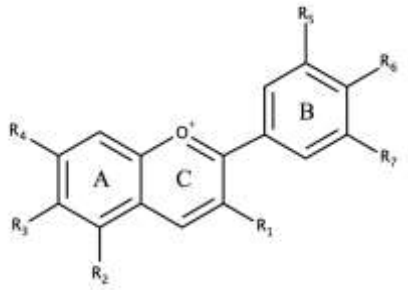

Figure 4.1 Backbone structure of ACNs

4.3.3 Metabolism

In general, ACNs are subjected to extensive metabolism after absorption, as recently demonstrated for cyanidin 3-glucoside (Ferrars et al., 2014). It has been suggested that their metabolites persist for longer periods of time in the circulation,

exerting their activities as long as their main molecular structure is maintained (Felgines et al., 2003; Pojer et al., 2013).

The mouth is often referred to as a benign environment for exogenous compounds that are ingested orally due to the short residence time in this cavity. However, researchers have showed that the hydrolysis of ACNs can occur in saliva and through oral microbial activity. Using a rinse solution containing ACNs, Mallery et al. (2011) reported the presence of aglycones in the mouth due to the β-glucosidase activity of enzymes produced by the host and oral microbiota. They also identified other key phase II enzymes, including uridine diphosphate-glucuronosyltransferases (UDPGT), catechol-*O*-methyltransferase (COMT), UDP-glucose dehydrogenase (UDPGDH), breast cancer resistance protein (BCRP), and lactase phlorizin hydrolase (LPH), in addition to sulfatase and β-glucoronidase reported by Chauncey, Lionetti, Winer, and Lisanti (1954). Comparing different rinse solutions, it was interesting to note that those containing flavouring agents resulted in lower salivary ACN levels, suggesting that the liquid was perceived as food by the sensory system, leading to release of saliva (confirmed by an increase in volume) and, consequently, β-glucosidase (Mallery et al., 2011). These results are in agreement with those by Kamonpatana et al. (2012), who showed that the ACN degradation promoted by saliva and buccal microbiota could be reduced by the use of the antibacterial chlorhexidine.

In the liver, phenolic aglycones are methylated by COMT (Galati, Lin, Sultan, & O'Briens, 2006) and/or conjugated with glucuronic acid (El Mohsen et al., 2006) or sulfate by UDPGT (Woodward et al., 2011) or phenol sulfotransferases (SULT), respectively (Felgines et al., 2003). According to Fleschhut, Kratzer, Rechkemmer, and Kulling (2006), ACNs and their aglycone forms are poor substrates for UDPGT, which would result in low glucuronidation rates and could explain the presence of intact ACNs in the circulation. These authors also indicated that ACNs and anthocyanidins are not substrates for enzymes of the cytochrome P450 family as they were not able to identify phase I metabolites *in vitro*. It has been suggested that sulfation predominates only when low doses of phenolic-derived drugs are administered, possibly because this pathway is easily saturated (Kay, 2006). Ferrars et al. (2014) recently identified several metabolites from cyanidin 3-glucoside in different body fluids.

Kay et al. (2005) presented the pharmacokinetics of ACNs after oral administration of a 721-mg dose of cyanidin 3-glycosides (galactoside, arabinoside, xyloside, and glucoside) from chokeberry extract in a small human trial consisting of healthy male volunteers (n = 3). This amount would be equivalent to 120–230 g of fresh whole berries. Unlike previous reports, approximately 32 % of the total amount of ACNs identified in the blood and urine consisted of the parent compounds while 68 % accounted for their respective metabolites (glucoronidated and methylated forms). Glucoronidation represented the main metabolic transformation, accounting for more than 50 % of the total ACNs detected in blood and urine, followed by methylation (approximately 48 %) (Kay et al., 2005).

Passamonti et al. (2009) noted that when ACNs and other flavonoids that undergo hepatic glucuronidation are ingested in high doses, this could lead to jaundice. Also that the antioxidant capacity of plasma which is commonly reported after their consumption would exhibit a transient and reversible increase in the concentration of circulating bilirubin, an endogenous antioxidant. This would be due to the ACNs and bilirubin competing for bilitranslocase in the liver, which may explain the slight increase observed in bilirubin after the administration of ACN-rich extract from elderberry in postmenopausal women in the study by Curtis et al. (2009).

4.3.4 Excretion

The type of metabolite (or conjugate) formed will determine the route of excretion of ACNs. For instance, glucuronides formed in the liver are readily available for biliary excretion unlike those formed in the intestine, which can reach and remain in the systemic circulation for a longer duration (higher $t_{1/2}$) (Kay, 2006). Fecal elimination can be reduced by enterohepatic circulation (EHC), which also extends the residence time of exogenous substances and their metabolites in the body (Kalt et al., 2014). The EHC can lead to the sulfation of compounds, diverting them from biliary excretion towards urinary excretion as highly polar compounds are not significantly reabsorbed by the intestine (Kay, 2006). The compounds that are not absorbed are hydrolyzed by colonic bacteria

and eliminated in the feces (Felgines et al., 2008; Fleschhut et al., 2006; Sánchez-Patán et al., 2012).

Values for fecal elimination are underestimated for most classes of polyphenols. In the case of ACNs, He et al. (2005) demonstrated that feces are one of the main routes for their excretion in rats. These results were confirmed by Felgines et al. (2010) and Czank et al. (2013), who assessed the fecal elimination of ACNs and their metabolites through the quantification of ^{14}C and ^{13}C isotope content in mice and humans, respectively. Czank et al. (2013) also showed that the content of ^{13}C isotope in the breath was approximately 6.9 ± 1.6 % of the initial dose, indicating that the lungs are also a route of excretion for ACN metabolites (Czank et al., 2013).

In relation to excretion via urine, He et al. (2009) showed that after stomach intubation of black raspberry extract, ACNs were detected in urine within 30 min. This observation may be explained by the presence of bilitranslocase in renal tissue at the basolateral membrane domain (Elias et al., 1990), which could indicate its involvement in the excretion of ACNs. Vanzo et al. (2008) demonstrated that the concentration of ACNs in the kidneys is three times higher than in the systemic plasma and liver, which corroborates the hypothesis of the transport being mediated by bilitranslocase. These results also indicated that the kidneys are more efficient in the uptake of ACNs than liver and, consequently, the excretion of these compounds would occur preferentially through the urine rather than bile (Vanzo et al., 2008, 2011). The localization of bilitranslocase in the basolateral and not in the apical domain suggests that the ACNs that eventually escape the glomerular filtration could be taken up into tubular cells and then excreted into the urine.

4.3.5 Bioavailability

When discussing PK parameters, the bioavailability is also an important aspect that should be explored. It refers to the rate and extent to which a drug reaches its site of action, i.e. the amount of compound ingested that will reach the systemic circulation and the specific organs or tissues, which will later result in a biological effect. Until recently, the bioavailability of ACNs was considered to be very low, although recent reports have

indicated that the data available from earlier studies in the literature could have underestimated ACN bioavailability since they did not monitor or considered the role of ACN metabolites (Kay, 2006). For example, Kalt et al. (2014) demonstrated that the bioavailability is higher than previously assumed and the elimination of metabolites continues for days after the consumption of ACNs has been discontinued, confirming the results obtained by Czank et al. (2013).

According to Ferrars et al. (2014), the conversion of cyanidin 3-glucoside into its metabolites, peonidin 3-glucoside and methoxy-cyanidin 3-glucoside-glucuronide, is reversible. Hwang, Kwan, and Albert (1981) indicated that in cases where a drug has a reversible metabolism (or biotransformation), the conventional concept of bioavailability and experimental strategies may not be adequate. This idea is relevant for the analysis of ACNs as both parent compounds and their metabolites can exhibit activity *in vivo*, and metabolites can be considered latent sources of active moiety, with prolonged effects (Hwang et al., 1981). EHC should also be considered a reversible metabolism in the study of ACN PK. When analyzing the results of absorption studies, proper consideration must be given to the role of different absorption site, as multiple peaks in the blood concentration do not necessarily indicate EHC. These multiple peaks could result from factors such as differences in carrier density and reduced gastric motility (Gabrielsson & Weiner, 2000).

In addition, blood AUC is commonly used to determine the bioavailability of exogenous compounds. However, according to Pastino and Conolly (2000) this strategy may not be appropriate for accurately estimating the bioavailability of compounds where their metabolism can be saturated. As discussed previously, the sulfation route is known to be easily saturated (Kay, 2006). It is also possible that technological constraints have limited the identification of sulfated metabolites, as reported by Walle, Hsieh, DeLegge, Oatis, and Walle (2004) for resveratrol metabolites. In addition, an increase in the amount ingested (or dosed) by 2-fold is not accompanied by the same increase in the AUC. For example, comparing the consumption of 250 and 500 g servings of microwave cooked purple carrot, researchers observed that the absorption of individual ACNs decreased with the larger dose (Novotny, Clevidence, & Kurilich, 2012), supporting the idea that the carrier molecules can also be saturated.

4.4 PHYSIOLOGICALLY-BASED PHARMACOKINETIC (PBPK) MODELLING OF ANTHOCYANINS

4.4.1 Motivation

The fate of ACNs *in vivo* can be described by using one- or multi-compartment models. In both situations, it is assumed that the compounds and their metabolites are linearly transported between a central compartment (e.g. blood and rapidly perfused tissues) and peripheral tissue compartments (e.g. slowly perfused tissues) (Chen & Gross, 1979). Determining the PK parameters of exogenous compounds and establishing an appropriate PBPK model is important for the following reasons: (a) it enables interspecies scale-up (e.g. data obtained in mice can be used to predict outcomes in humans) (Bradshaw-Pierce, Eckhardt, & Gustafson, 2007); (b) it provides an association between the concentration of the compound, effects, and responses, which can help in determining the dose regimen; and (c) it can help in elucidating the effects of disease states on the PK of the compound (e.g. different blood flow rates can be used to better mimic a pathological condition *in vivo*).

As discussed previously, one-compartment models offer limited information about the PK of ACNs and their metabolites after ingestion. In addition, it takes some time for the compounds to distribute into the organs and tissues and reach equilibrium, which makes the one-compartment model unsuitable for exogenous compounds where concentration is measured frequently (Gabrielsson & Weiner, 2000). On the other hand, the PBPK multi-compartment model is much more comprehensive and accounts for the anatomic site of absorption, metabolism, distribution, and excretion of the exogenous compounds, as well as factors such as the blood flow rates, physiologic organ or tissue volumes, and tissue binding (Chen & Gross, 1979). This model consists of differential equations for each compartment, where a mass balance and the following assumptions are observed: (1) the model is flow-limited (i.e. it is assumed that the concentration of the exogenous compound in the arterial blood reaches rapid equilibrium with each compartment and concentrations in each compartment would be proportional to that in the venous blood leaving the organ/tissue); (2) the plasma protein and tissue binding are

linear (i.e. the coefficient of distribution ratio or partition coefficient of the compound between the tissue and plasma is independent of the concentration of the compound); and (3) concentration of the exogenous compound is homogeneous in a given compartment (Chen & Gross, 1979).

Boonpawa, Spenkelink, Rietjens, and Punt (2014) recently described a PBPK model for quercetin and its metabolites to predict the time-dependent concentrations of these compounds in plasma and other compartments. These authors reported that the model was adequate in predicting biliary and urinary excretion and intestine efflux of quercetin metabolites through the quantification of these compounds (Boonpawa et al., 2014). Although their proposed model could be used for any flavonoid, it is lacking a separate compartment for the stomach, which may be a significant omission given the role of the stomach in the absorption of ACNs.

In the following sections, a comprehensive theoretical PBPK model is first developed for the parent ACNs and then a separate PBPK model is developed for their metabolites. The equations for each compartment are presented separately to better explain the model. For a comprehensive review and summary of appropriate values to use for some of the non-specific variables common to other models, the reader is referred to the work by Brown, Delp, Lindstedt, Rhomberg, and Beliles (1997).

4.4.2 Multi-Compartmental PBPK Model for Parent Anthocyanins

The PBPK model for ACNs that is proposed in this study consists of nine separate compartments, representing the organs or tissues that are involved in the ADME of the parent compounds (Figure 4.2). These compartments are the stomach, small intestine, large intestine, liver, kidney, slowly perfused tissues, rapidly perfused tissues, lungs, and blood, where arrows are used in Figure 4.2 to indicate the movement of the digestive bolus through the digestive tract, connecting the compartments for the stomach and intestines. In this model, it is also assumed that blood from the hepatic artery and the portal vein mix in the sinusoids before entering the liver (Kan & Madoff, 2008), and that the concentration of the compound in the tissue is proportional to the outgoing venous concentration of the parent ACN (Wagner, 1993). It is worth noting that the aglycone

anthocyanidins are not included in the discussion of the model as their occurrence in nature is still not completely characterized (Macz-Pop, Rivas-Gonzalo, Pérez-Alonso, & González-Paramás, 2006).

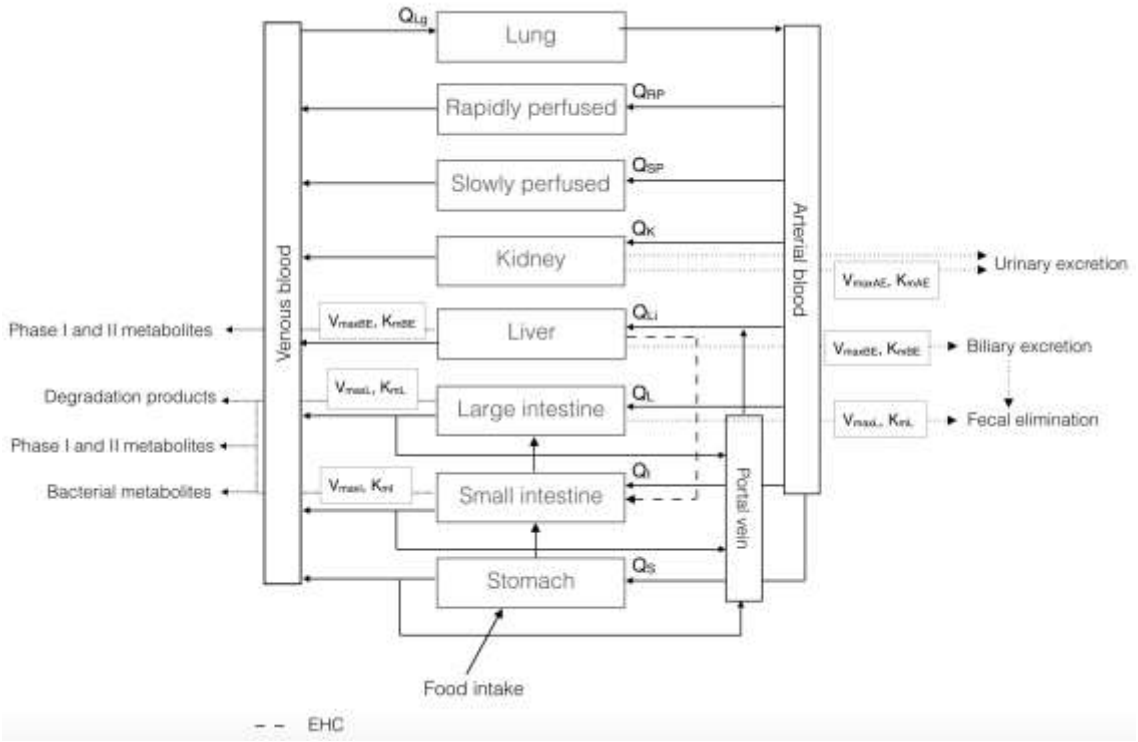

Figure 4.2 Multi-compartmental PBPK model for parent ACN

The dotted arrows to the right of large intestine, liver, and kidneys indicate loss of the parent ACNs as a result of elimination or excretion, whereas those to the left of the small and large intestines and liver indicate loss of the parent ACNs by metabolism (from microbiota or host). A fraction of these metabolites is excreted and the remainder is absorbed. In the case of the kidneys, there are two arrows leaving this compartment indicating passive and active urinary excretion. Further details regarding absorption of the metabolites are given in the sub-compartment model for metabolites described in section 4.4.3. The model equations for the different compartments shown in Figure 4.2 are presented below. These equations were developed from other studies in the literature, such as those described by Boonpawa et al. (2014) for quercetin.

Stomach. The first site for ACN absorption in the body is the stomach (Cai et al., 2011; Felgines et al., 2007). The equations for this compartment consider the ACNs that would

be bioaccessible, accounting for food that is ingested and/or digested over time, along with the bolus that is transferred to the small intestine. In addition, the quantity of ACNs being absorbed and circulated through the gastric tissue via blood is included. Even though it has been suggested that passive diffusion and absorption involving the bilitranslocase carrier could occur simultaneously in the stomach depending on the ACN structure and gastric conditions (Fernandes et al., 2012), in this model they were combined into one term in the equation.

$$A_{St\_Lu_i} = A_{F_i} - dA_{I\_Lu_i} \tag{1}$$

$$\left(dA_{St_i}/dt\right) = K_{a_{St_i}} A_{St\_Lu_i} + Q_{St}\left(C_{B_i} - {C_{St_i}}/{P_{St:B_i}}\right) \tag{2}$$

$$C_{St_i} = \left({A_{St_i}}/{V_{St}}\right) \tag{3}$$

Small Intestine. In addition to stomach, the small intestine constitutes an important organ for absorption of ACNs (Talavéra et al., 2004), even though its pH could lead to the chemical degradation of these compounds (He et al., 2009). Similar to stomach, the equations that are described take into account the amount of bolus that enters the small intestine and is transferred to the large intestine over time, as well as the quantity that is absorbed or eliminated through metabolism, either by the host or microbiota. It is possible that different isoenzymes are used to metabolize the ACNs, in which case, the model would need to account for this with different values of K_m.

$$A_{I\_Lu_i} = \left(A_{F_i} - A_{St_i}\right) - A_{L\_Lu_i} \tag{4}$$

$$\left(dA_{I_i}/dt\right) = K_{a_{I_i}} A_{I\_Lu_i} + Q_I\left(C_{B_i} - {C_{I_i}}/{P_{I:B_i}}\right) - \left[\left(\frac{V_{max,I\_mi}\,{C_{I_i}}/{P_{I:B_i}}}{K_{m,I\_mi} + {C_{I_i}}/{P_{I:B_i}}}\right)_{metab} + \left(\frac{V_{max,I\_mi}\,{C_{I_i}}/{P_{I:B_i}}}{K_{m,I\_mi} + {C_{I_i}}/{P_{I:B_i}}}\right)_{microb}\right] \tag{5}$$

$$C_{I_i} = \left({A_{I_i}}/{V_I}\right) \tag{6}$$

where *mi* is the metabolite; *metab* and *microb* indicate the metabolism of the compound by the host and microbiota, respectively.

Another important consideration is the EHC that could contribute to the concentration of ACNs in the intestine. In this post-absorption situation, the model would have a term to account for the amount of ACNs from the liver via biliary excretion $\left(Q_{Li}\left({}^{C_{Li_i}}\!/_{P_{Li:B_i}}\right)\right)$. Then, equation 6 would be expressed as $C_{I_i} = \left({}^{A_{I_i} + A_{I_{i\_EHC}}}\!/_{V_I}\right)$, where $A_{I_{i\_EHC}}$ represents the amount of parent ACN that is being circulated in the EHC.

Large Intestine. The equations for the large intestine are very similar to those for the small intestine compartment, however the amount that will be eliminated through the feces is also considered here. Feces comprise of approximately 25 % solids, from which 30 % are undigested material associated with bile salts (Barbosa, 2013). It is possible that ACNs (and their metabolites) could interact with these compounds. For example, Aura et al. (2005) showed that the recovery of ACNs from spiked feces was incomplete, which could indicate that these compounds are able to bind to the fecal matrix. Feces seem to be the main vehicle of elimination of ACNs (He et al., 2005), even though most results have been underestimated.

$$A_{L\_Lu_i} = A_{F_i} - (A_{St_i} + A_{I_i}) - A_{Feces_i} \tag{7}$$

$$\left(dA_{L_i}/dt\right) = Q_L\left(C_{B_i} - {}^{C_{L_i}}\!/_{P_{L:B_i}}\right) - \left[\left(\frac{V_{max,L\_mi}\,{}^{C_{L_i}}\!/_{P_{L:B_i}}}{K_{m,L\_mi} + {}^{C_{L_i}}\!/_{P_{L:B_i}}}\right)_{metab} + \left(\frac{V_{max,L\_mi}\,{}^{C_{L_i}}\!/_{P_{L:B_i}}}{K_{m,L\_mi} + {}^{C_{L_i}}\!/_{P_{L:B_i}}}\right)_{microb}\right] \tag{8}$$

$$C_{L_i} = \left({}^{A_{L_i}}\!/_{V_L}\right) \tag{9}$$

Liver. Parent ACNs are subjected to extensive metabolism in the liver. In this model, the clearance of parent ACNs through biliary excretion is included. In addition, the model accounts for the concentration of ACNs in the liver from the ingested food along with the amount circulated in the EHC, as already shown for the small intestine.

$$\left(dA_{Li_i}/dt\right) = Q_{St}\left({C_{St_i}}/{P_{St:B_i}}\right) + Q_I\left({C_{I_i}}/{P_{I:B_i}}\right) + Q_L\left({C_{L_i}}/{P_{L:B_i}}\right) + Q_{Li}\left(C_{B_i} - {C_{Li_i}}/{P_{Li:B_i}}\right) -$$

$$\left[\left(\frac{V_{max,BE\_mi}\,{C_{Li_i}}/{P_{Li:B_i}}}{K_{m,BE\_mi} + {C_{Li_i}}/{P_{Li:B_i}}}\right)_{metab} + \left({CL'}_{Li_i} f_{B_i} {C_{Li_i}}/{P_{Li:B_i}}\right)\right] \quad (10)$$

$$C_{Li_i} = \left({A_{Li_i}}/{V_{Li}}\right) \quad (11)$$

Kidneys. ACNs and their metabolites have been identified in the urine, which is the biological fluid commonly assessed to determine the bioavailability of these compounds. Using a similar approach to that used for the liver compartment, a term is included for the renal clearance of parent ACNs through urine. In addition, a term is also included to account for active renal excretion (AE) of the compounds that eventually escape the glomerular filtration, since bilitranslocase was also identified in the basolateral membrane domain in the renal tissue (Elias et al., 1990).

$$\left(dA_{K_i}/dt\right) = Q_K\left(C_{B_i} - {C_{K_i}}/{P_{K:B_i}}\right) - \left[\left(CL_{K_i} f_{B_i}\,{C_{K_i}}/{P_{K:B_i}}\right) + \left(\frac{V_{max,AE}\,{f_{B_i} C_{Ki_i}}/{P_{K:B_i}}}{K_{m,AE} + {f_{B_i} C_{Ki_i}}/{P_{K:B_i}}}\right)\right] \quad (12)$$

$$C_{K_i} = \left({A_{K_i}}/{V_K}\right) \quad (13)$$

$$CL_{K_i} = k_e V_i \quad (14)$$

Slowly and Rapidly Perfused Tissues. Examples of slowly perfused tissues include the skin and muscles, whereas the brain and heart would be categorized as rapidly perfused organs. They were included in this model since research has shown that they are sites for distribution and accumulation of parent ACNs and their metabolites (Andres-Lacueva et al., 2005; Kalt et al., 2008a). Equations 15 and 16 refer to slowly perfused tissues, whereas equations 17 and 18 are for the rapidly perfused compartment.

$$\left(dA_{SP_i}/dt\right) = Q_{SP}\left(C_{B_i} - {C_{SP_i}}/{P_{SP:B_i}}\right) \quad (15)$$

$$C_{SP_i} = \left({A_{SP_i}}/{V_{SP}}\right) \quad (16)$$

$$\left(dA_{RP_i}/dt\right) = Q_{RP}\left(C_{B_i} - {}^{C_{RP_i}}\!/_{P_{RP:B_i}}\right) \quad (17)$$

$$C_{RP_i} = \left({}^{A_{RP_i}}\!/_{V_{RP}}\right) \quad (18)$$

Lungs. Although the lungs are not involved in the elimination of the parent ACNs, a separate compartment was included in this model as a site for converting venous blood into arterial blood, which will ultimately be distributed to other compartments.

$$\left(dA_{Lg_i}/dt\right) = Q_{Lg}\left({}^{C_{Lg_i}}\!/_{P_{Lg:B_i}} - C_{B_i}\right) \quad (19)$$

$$C_{Lg_i} = \left({}^{A_{Lg_i}}\!/_{V_{Lg}}\right) \quad (20)$$

Blood. The concentration of parent ACNs in the blood is the sum of the concentration of the various compartments in the model, taking into account the blood flow rate in these organs/tissues and the partition coefficient of the compounds, in addition to the cardiac output (although the heart is not considered a separate compartment). As discussed previously, parent ACNs can be bound to different components in the blood, such as HSA (Wiese et al., 2009), and there is an equilibrium of the free and bound form of these compounds.

$$\left(dA_{B_i}/dt\right) = \left[(Q_{St} + Q_I + Q_L)\left({}^{C_{Li_i}}\!/_{P_{Li:B_i}}\right) + Q_K\left({}^{C_{K_i}}\!/_{P_{K:B_i}}\right) + Q_{SP}\left({}^{C_{SP_i}}\!/_{P_{SP:B_i}}\right) + Q_{RP}\left({}^{C_{RP_i}}\!/_{P_{RP:B_i}}\right) + Q_{Lg}\left({}^{C_{Lg_i}}\!/_{P_{Lg:B_i}}\right)\right] - (Q_C C_{B_i}) \quad (21)$$

$$C_{B_i} = \left({}^{A_{B_i}}\!/_{V_B}\right) \quad (22)$$

4.4.3 Sub-Compartmental Model for Metabolites^n:

This model, shown schematically in Figure 4.3, is very similar to the one proposed for the parental ACNs in section 4.4.2, where the notation ^{n} indicates that the compartmental model can be used for *n* generations of metabolites. The differences are:

(a) the absence of a compartment for stomach, since it is considered that the generation of the metabolites occurs after ingestion due to the limited residence time in the mouth and stability in the gastric environment, (b) and inclusion of a compartment for the lungs as some metabolites can be eliminated by this route (through breath) (Czank et al., 2013). As all other compartments have been previously described in the model for the parent ACNs, only the equations for the lung compartment are given in this section for the metabolite model.

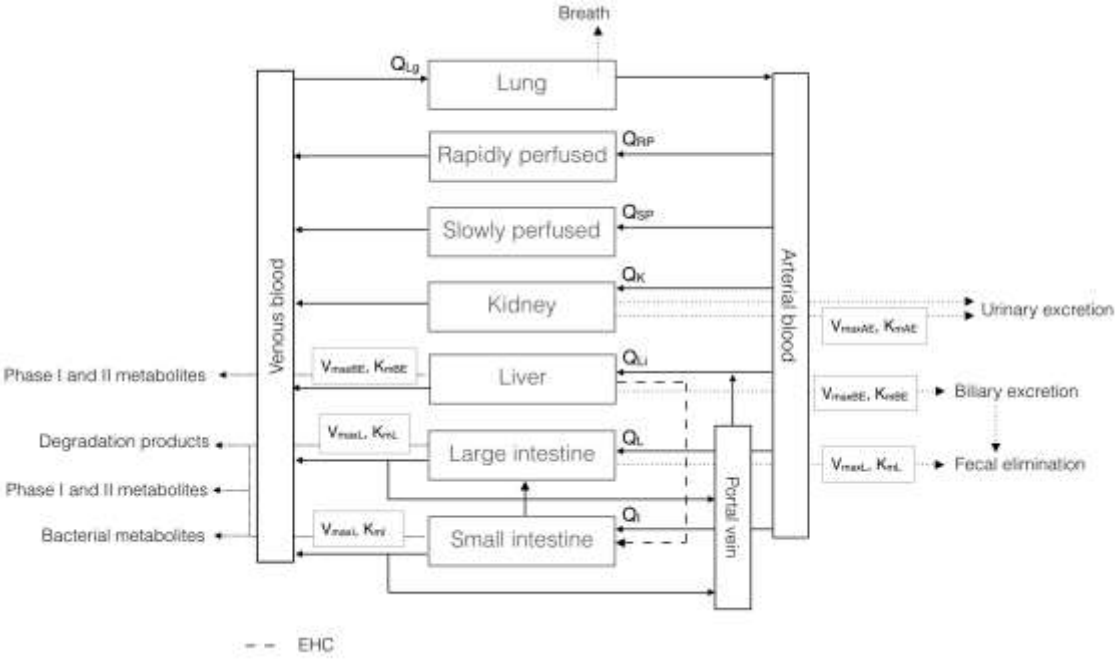

Figure 4.3 PBPK compartment-model for metabolites

Lungs. ACN metabolites have been shown to be eliminated through the breath (Czank et al., 2013), in a similar way to other flavonoids. In the case of quercetin, approximately 52 % of an isotope-labeled compound administered to healthy human volunteers was recovered in respiratory carbon dioxide (Petrakis, Kallianos, Wender, & Shetlar, 1959; Walle, Walle, & Halushka, 2001).

$$\left(dA_{Lg_{mi}}/dt\right) = Q_{Lg}\left({}^{C_{Lg_{mi}}}\!/_{P_{Lg:B_{mi}}} - C_{B_{mi}}\right) - Q_v\left[{}^{C_{Lg_{mi}}}\!/_{\left(P_{Lg:B_{mi}}P_{B:Air_{mi}}\right)}\right] \quad (23)$$

$$C_{Lg_{mi}} = \left({}^{A_{Lg_{mi}}}\!/_{V_{Lg}}\right) \quad (24)$$

where Q_v is the alveolar ventilation; $P_{B:Air}$ is the blood:air partition coefficient.

4.4 CONCLUSION

Overall, the theoretical PBPK models developed in this study highlight the complex mechanisms involved in the absorption, distribution, metabolism, and excretion of parent ACNs and their metabolites *in vivo*. These models could be used to predict the plasma concentrations of these compounds and provide an indication of their fate after ingestion, based on results obtained in preclinical (i.e. *in vitro* and animal trials) and clinical research. The ability of the models to accurately simulate and estimate the concentrations of circulating compounds still has to be determined.

Chapter 5 GASTRORETENTIVE SYSTEMS – A PROPOSED STRATEGY TO MODULATE ANTHOCYANIN RELEASE AND ABSORPTION FOR THE MANAGEMENT OF DIABETES

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5.1 ABSTRACT

Several reports have indicated a positive correlation between the consumption of anthocyanins (ACNs) and biomarkers relating to the improvement of type 2 diabetes (T2D). However, the results from *in vitro* studies often do not translate into clinical evidence. Potential causes of these discrepancies are experimental conditions that lack physiological relevancy; extensive degradation of these compounds *in vivo* due to changes in pH and metabolism; and a short residence time in the absorption window in relation to the absorption rate. Here, gastroretentive systems (GRS) are proposed as a strategy to overcome the limitations in ACN delivery and to reduce the existing bench-to-subject gap. This review summarizes recent literature on the use of ACNs for the management and control of T2D, followed by GRS platforms to promote a sustained release of ACNs for increased health benefits.

5.2 INTRODUCTION

Fruits are a rich source of essential micronutrients (such as vitamins and folic acid) (Brouwer et al., 1999; Proteggente et al., 2002) and other non-essential bioactive compounds, including anthocyanins (ACNs) (Faramarzi et al., 2015; Fischer, Jaksch, Carle, & Kammerer, 2013). ACNs are members of the flavonoid class of secondary metabolites and important hydrophilic pigments in plants (Delgado-Vargas, Jiménez, & Paredes-López, 2000). In addition to their colorant properties, several studies have

indicated a positive association between the consumption of ACNs and reduced risk of degenerative diseases (Cassidy et al., 2011, 2013; Pascual-Teresa, Moreno, & García-Viguera, 2010; Wallace, 2011; Wang & Stoner, 2008), including type 2 diabetes (T2D) (Guo & Ling, 2015; van Dam, Naidoo, & Landberg, 2013).

Nonetheless, there is still a considerable gap between the results obtained *in vitro* and those confirmed *in vivo*. One explanation is that the health-promoting effects are often from *in vitro* and/or *in vivo* studies, where the ACN compounds are administered in quantities that are above those commonly ingested through diet or that would be observed *in vivo* (Al-Awwadi et al., 2004; Heo & Lee, 2005; Hidalgo et al., 2012; Kelsey, Hulick, Winter, Ross, & Linseman, 2011; Tarozzi et al., 2007, 2010). It is also possible that the experiments do not account for the accumulation and synergic effect of the compounds ingested and the metabolites produced *in vivo* (Kelsey et al., 2011). In addition to experimental design that has little physiological relevancy, another possible reason for this gap could include the extensive degradation of ACNs due to variations in pH or as a result of metabolism, as demonstrated for cyanidin 3-glucoside (Ferrars et al., 2014).

The insufficient residence time in the upper GI tract (stomach and initial portion of the small intestine) could be a factor that limits the absorption of the parent ACN, as it would contribute to degradation at higher pHs and due to metabolism. In this context, gastroretentive systems (GRS) are a promising strategy that could help to increase the retention time and modulate the release of ACNs in portions of GI tract where they are absorbed. GRS differ from conventional delivery systems as the time they remain in the stomach is higher, prolonging the residence time of the bioactive compound in this organ (Joseph, Lakshmi, & Jayakrishnan, 2002). For substances absorbed in the stomach, these systems can assist in increasing their absorption and improving their bioavailability. The development of a GRS can be achieved by different strategies that will be described in this review. Since these platforms have not been explored in depth by the food industry, information from the pharmaceutical sciences will be used as a reference when appropriate, then recommendations will be made for GRS for fruit-derived extracts that are rich in ACNs and have potential as health-promoting compounds.

The purpose of this review is to present the use of GRS as a potential platform to modulate the release of ACNs in their absorption window (upper GI tract) to assist in the

management of degenerative diseases. Firstly, the evidence that correlates ACNs with the management of degenerative diseases (using T2D as an example) is discussed. Then, the role of the stomach in the absorption of ACNs is presented, followed by different classes of GRS based on information available from the pharmaceutical sciences and the potential application of GRS for the management of T2D.

5.3 ANTHOCYANINS IN THE MANAGEMENT OF TYPE 2 DIABETES (T2D)

Diabetes currently affects approximately 387 million people worldwide and it is estimated that this number will rise to 592 million by 2035 (International Diabetes Federation, 2013). T2D is characterized by insulin resistance and relative lack of insulin secretion, and accounts for more than 90 % of the cases reported (Kahn, 1998). Insulin plays a key role with glucagon in regulating the concentration of glucose in the blood and is secreted by β-cells of the pancreatic islets of Langerhans as a response to high levels of glucose and fatty acids in the circulatory system (Gravena, Mathias, & Ashcroft, 2002). Glucagon, on the other hand, is secreted by α-cells of these islets, promoting the glycolysis of glycogen in the liver and an increase in blood glucose (Foster, Leapman, Li, & Atwater, 1993). Ishihara, Maechler, Gjinovci, Herrera, and Wollheim (2003) indicated that the activation of β-cells leads to a suppression of the α-cells. Current treatment agents available for T2D include insulin, sulfonylureas, and α-glucosidase inhibitors (Nathan et al., 2009). However, adverse effects have been commonly associated with these drugs, such as hypoglycemia (Krepinsky, Ingram, & Clase, 2000), weight gain (Nathan et al., 2009), and drug-drug interactions observed with the use of sulfonylureas (Schelleman et al., 2014). Research indicates that the consumption of polyphenols, such as ACNs, can be associated with an improvement of biomarkers of T2D (Table 5.1), which could help with the prevention and management of diabetes.

Table 5.1 Summary of anti-diabetic activities promoted by ACN from different sources

Source	Model	Activity	Reference
In vitro			
Blueberry (fruit)	β-cells	↑ proliferation	Martineau et al., 2006
Cornus fruit (extract)	β-cells	↑ insulin secretion	Jayaprakasam, Vareed, Olson, & Nair, 2005
Maqui berry	Myotubes	↑ glucose uptake Insulin-sensitizing effect	Rojo et al., 2012
Maqui berry	H4IIE cells	↓ glucose production Insulin-sensitizing effect	Rojo et al., 2012
In vivo			
Bilberry (powder)	Rat, alloxan-induced diabetes	↓ blood glucose levels ↑ serum insulin levels	Asgary, RafieianKopaei, Sahebkar, Shamsi, & Goli-malekabadi, 2016
Black rice (extract)	Rat, fructose diet	Improved glucose intolerance No effect on hyperinsulinemia	Guo et al., 2007
Cornelian cherry (purified ACNs)	Mice C57BL/6, high fat diet	↓ weight gain independent of food intake ↑ insulin levels Improved glucose tolerance Protection of islets	Jayaprakasam, Olson, Schutzki, Tai, & Nair, 2006
Cyanidin 3-glucoside	Mice KK-A^{y}	↓ blood glucose Improved insulin sensitivity	Sasaki et al., 2007
Gamazumi (extract)	Rat, streptozotocin-induced diabetes	↓ blood glucose levels ↓ hemoglobin A1c	Iwai, Onodera, & Matsue, 2004
Haskap berry	SD/SPF rats, high fat diet	↓ postprandial blood glucose	Takahashi et al., 2014
Lowbush blueberry (ACN-rich formulation)	Mice C57BL/6J	↓ blood glucose levels	Grace et al., 2009
Maqui berry	Rat, diabetic	↓ blood glucose and glucose tolerance	Rojo et al., 2012

Source	Model	Activity	Reference
Purple corn	Mice, high fat diet	Hyperglycaemia and hyperinsulinemia normalized after high fat diet	Tsuda, Horio, Uchida, Aoki, & Osawa, 2003
Purple sweet potato (extract)	Sprague-Dawley rat	↓ postprandial glycaemic response and rise of blood glucose levels	Matsui et al., 2002
Purple-fleshed potato	Rat, streptozotocin-induced diabetes	↓ blood glucose levels ↑ serum insulin levels	Choi, Park, Eom, & Kang, 2013
Red wine (Cabernet)	Rat, streptozotocin-induced diabetes	↓ blood and urine glucose Inhibition of body mass loss ↓ lipid peroxidation	Jankowski, Jankowska, & Niedworok, 2000
Tart cherry	Rat, insulin resistance	↓ blood glucose, cholesterol, and insulin	Seymour et al., 2008
Clinical			
Cranberry (juice concentrate powder)	T2D patients	No differences were found between placebo and treatment group	Chambers & Camire, 2003
Muscadine grape (wine)	T2D patients	↓ blood glucose, insulin Glycaemic control	Banini, Boyd, Allen, Allen, & Sauls, 2006
Sour cherry (concentrated juice)	T2D patients	↓ body weight, hemoglobin A1c	Ataie-Jafari, Hosseini, Karimi, & Pajouhi, 2008

5.3.1 *In Vitro* Evidence

As highlighted in Table 5.1, there are *in vitro* studies associating ACNs with anti-diabetic properties. Rojo et al. (2012) showed that ACN-rich extract from maqui berry could ameliorate diabetic conditions *in vitro* by reducing the production of glucose and increasing its cellular uptake. The authors used two systems to assess glucose production and uptake: H4IIE rat hepatoma cells and myotubes (differentiated from L6 myoblasts isolated from rat skeletal muscles). H4IIE cells are frequently used for the study of gluconeogenesis and as an *in vitro* model for obesity-related insulin resistance (T2D). The physiological relevance of these cells lies with their production of glucose, which is stimulated *in vitro* by glucagon and glucocorticoid simulators, and inhibited by insulin, in

similar ways to what occurs *in vivo* (Hectors, Vanparys, Pereira-Fernandes, Knapen, & Blust, 2012). Myotubes, on the other hand, are often used to evaluate the uptake of glucose (Nedachi & Kanzaki, 2006). When stimulated by insulin, skeletal muscle cells translocate the glucose transporter 4 (GLUT4) to the plasma membrane to enable glucose uptake, and this is observed also in the myotube model (Galante et al., 1995). Rojo et al. (2012) found that ACN glycosides present in the maqui berry extract had a mild effect on the downregulation of glucose-6-phosphatase (G6Pase) gene compared to insulin and also observed that the concomitant administration of ACNs and sub-optimal dose of insulin had a higher effect on the downregulation of G6Pase gene. G6Pase is involved in glucose homeostasis and its expression is strongly inhibited by insulin (Chen, Meseck, McEvoy, & Woo, 2000). They reported a significant increase of glucose uptake in the cells treated with ACNs and also those receiving ACNs and insulin. From this evidence, they suggested that ACNs were sensitizing the cells to insulin, resulting in the improved glucose uptake that was observed (Rojo et al., 2012).

In a different study, Jayaprakasam et al. (2005) evaluated the properties of purified ACN glycosides and aglycones as insulin secretagogues in β-cells from rodents. Among the compounds investigated, purified glucosides of cyanidin and delphinidin were the most effective in inducing insulin secretion. It is important to note that ACNs can be extensively metabolized by the gut microflora prior to absorption (Fang, 2014; Faria, Fernandes, Norberto, Mateus, & Calhau, 2014), and this was not considered in the *in vitro* studies by Rojo et al. (2012) and Jayaprakasam et al. (2005). This can impact the significance of these findings, for example it is unlikely that ACN glycosides will reach the pancreatic β-cells in their native form and exhibit the health-promoting effects *in vivo*.

5.3.2 *In Vivo* Evidence

Animal models have been frequently used to investigate the effect of ACNs on diabetes-related conditions. For example, Rojo et al. (2012) evaluated the hypoglycaemic effect and glucose tolerance in obese hyperglycaemic C57BL/6J mice after administering ACN-rich extract from maqui berry. C57BL/6J mice develop T2D when treated with a high fat, high-single-carbohydrate, low-fibre diet due to a genetic predisposition (Surwit,

Kuhn, Cochrane, McCubbin, & Feinglos, 1988). In this model, ACNs administered orally improved blood glucose concentrations and glucose tolerance and this effect was partially attributed to delphinidin 3-sambubioside-5-glucoside (Rojo et al., 2012). Other studies using C57BL/6J mice have shown that ACN has a positive effect in controlling weight gain, which would contribute to the management of T2D through the consumption of ACNs from haskap berries (Wu et al., 2013).

In the study by Takikawa, Inoue, Horio, and Tsuda (2010), a positive effect on blood glucose and insulin sensitivity was observed in diabetic KK-A^y rats consuming bilberry extract for five weeks. These authors corroborated the *in vitro* results obtained by Martineau et al. (2006) indicating that the effect of ACNs would be through an insulin-independent mechanism.

In both models presented (C57BL/6J and KK-A^y mice), the animals were genetically predisposed for developing T2D-like conditions. Diabetes can also be chemically-induced (by alloxan or streptozotocin) to simulate diabetes caused by insulin deficiency. Chemical treatment results in the loss of β-cell mass and immunological activity in the islets (Zunino, 2009). Examples of studies that used a chemically-induced diabetes model are provided in Table 5.1.

5.3.3 Clinical and Epidemiological Evidence

Studies conducted on healthy human volunteers have shown that the consumption of berries could be associated with an improvement of the postprandial plasma glucose, which can reduce the risks of developing T2D (Törrönen et al., 2010, 2012b; Törrönen, Kolehmainen, Sarkkinen, Mykkänen, & Niskanen, 2012a). In a cross-over study (randomized, single-blinded, 5-day wash-out period), the consumption of a sucrose-sweetened berry purée (prepared with bilberry, blackcurrant, cranberry, and strawberry) was compared to a control consisting only of sucrose. Plasma glucose levels were significantly lower in the first 30 min after ingestion of the berry purée compared to the sucrose control. There was also a delay to reach the maximum plasma glucose concentration: 45 and 30 min for the berry puree treatment and control meal, respectively, which could indicate a reduction in digestion and/or absorption of sucrose similar to *in*

vivo results (Törrönen et al., 2010). However, the berry purée was not assessed for its polyphenol content. Based on literature values, the authors estimated that ACNs were one of the major polyphenolic component in the berry puree; however, other compounds could have contributed to these results.

In a double-blind placebo-controlled trial, Stull et al. (2010) evaluated the effect of blueberry consumption in 32 obese, non-diabetic, and insulin-resistant subjects. Although these volunteers were not diabetics, they presented high risk for development of T2D. The subjects were divided randomly into two groups: (a) treatment, consisting of freeze-dried blueberry powder (22.5 g) in a smoothie, and (b) placebo, consisting of a smoothie with equal macronutrient content and without blueberry powder. The smoothies were consumed twice a day (breakfast and dinner) for six weeks, and those containing blueberry powder represented approximately a cup of fresh whole berries per smoothie. The authors observed a significant improvement on the insulin sensitivity in subjects consuming the smoothie containing blueberries (Stull et al., 2010).

In a study with obese T2D patients, the consumption of a capsule containing concentrated bilberry extract reduced the area under curve (AUC) of glucose and insulin in comparison to a placebo. This double-blind, cross-over study consisted of: (a) treatment with one gelatine capsule filled with bilberry extract (containing 36 % ACNs), representing 50 g of fresh berries, and (b) placebo consisting of a capsule with microcrystalline cellulose (Hoggard et al., 2013). After ingesting the capsule with berry extract or placebo, the volunteers consumed a drink with 75 g of glucose and were evaluated for glucose metabolism, with a two-week wash-out period. The authors suggested that the reduction in the AUC could be related to a reduction of the carbohydrate digestion and/or absorption. In addition, they reported a reduction of plasma insulin levels, possibly as a result of lower plasma glucose (reduction of approximately 18 % of the AUC compared to the placebo) or due to an improvement on insulin sensitivity (Hoggard et al., 2013). A limitation of this study is the small number of participants enrolled (eight). However, it is the first demonstration of the beneficial effects of consuming a concentrated bilberry extract on T2D management.

Li, Zhang, Liu, Sun, and Xia (2015) also showed that the consumption of 160 mg of ACNs purified from bilberries and blackcurrants split into two portions daily for 24

weeks had positive effects on 58 diabetic patients. Patients showed a reduction in fasting plasma glucose in comparison to the placebo group, in addition to improvement of dyslipidaemia.

In relation to epidemiological studies, Samieri, Sun, Townsend, Rimm, and Grodstein (2014) studied the ingestion of different classes of flavonoids by 13,818 healthy women (> 50 years old) for 15 years in an observational study, using questionnaires. Of those individuals who survived into their seventies, 1,517 met the requirement of healthy aging (i.e. no chronic disease; high degree of cognitive, mental, and physical health) and the higher consumption of flavonoids, including ACNs, was associated with higher chances of health and wellbeing at older ages (Samieri et al., 2014).

Wedick et al. (2012) also showed that higher intakes of ACNs were inversely related to the development of T2D using data from three cohort studies consisting of more than 200,000 healthy subjects. The effect of other classes of flavonoids was not consistent between the cohort studies, which could indicate that they made little contribution to the reduced risk of T2D (Wedick et al., 2012). In addition, Muraki et al. (2013) suggested that a higher risk of T2D could be associated with the higher consumption of fruit juices, possibly because of the higher glycaemic load and degradation of bioactive compounds during processing.

5.4 Possible Use of Gastroretentive Systems for the Management of Type 2 Diabetes

Several reports have indicated a positive association between the consumption of ACNs, reduced risk, and management of T2D (Hoggard et al., 2013; Muraki et al., 2013; Samieri et al., 2014; Stull et al., 2010; Törrönen et al., 2010, 2012a, 2012b; Wedick et al., 2012). However, most studies presented in this review used complex fruit extracts that contain several ACNs in addition to other plant metabolites. For instance, Takikawa et al. (2010) identified 15 ACNs in the bilberry extract used in their study. In a different study, Rojo et al. (2012) isolated and identified delphinidin 3-sambubioside-5-glucoside as the ACN partially responsible for improvement of glucose tolerance and blood concentration.

However, this compound represented only 6.76 % of the total ACNs in the extract. In addition, it should be noted that the doses used in *in vitro* and *in vivo* studies with rodents to investigate the health-promoting effects of ACNs are often above the quantities commonly consumed in a normal human diet, as in the study by Al-Awwadi et al. (2004), Heo and Lee (2005), Hidalgo et al. (2012), and Tarozzi et al. (2007, 2010).

The extrapolation of the results obtained *in vitro* are also limited, even though they can provide some indication of the mechanisms involved in the antidiabetic properties exhibited by polyphenols. ACNs are highly metabolized *in vivo*, and it is unlikely that the compounds that exert the activities are the ones found in the fruit. For example, Ferrars et al. (2014) recently identified 25 metabolites of cyanidin 3-glucoside in body fluids. Thus, the presence of numerous ACNs (and other polyphenols) in the same extract can contribute to the complexity involved in establishing the association.

The specific absorption of ACN glycosides, which are the forms of ACNs found in foods and studied *in vitro*, could be increased by prolonging their residence time in the upper GI tract and by providing a means for controlled release, which would also contribute to reduce their degradation due to changes in pH and metabolism.

ACNs are generally stable under the acidic environment of the stomach (pH < 2) (He et al., 2009; Liang, et al., 2012; Liu et al., 2014; McDougall, Dobson, Smith, Blake, & Stewart, 2005; Stalmach, Edwards, Wightman, & Crozier, 2012) as they are likely to be found in the stable flavylium cation form (Brouillard & Dubois, 1977). Although the stomach is not often viewed as an absorption site, research has suggested that it could have a role in the absorption of ACNs (Felgines et al., 2007), which would help to explain the rapid detection of these compounds in the plasma (Vanzo et al., 2011). For example, the *in situ* gastric administration of purified ACNs extracted from blackberry and bilberry in anesthetized Wistar rats for 30 min resulted in the absorption of approximately 25 % of the ACN monoglycosides (cyanidin and malvidin glucoside or galactoside). Cyanidin 3-glucoside metabolites were identified in the bile within 20 min (Talavéra et al., 2003). The study by He et al. (2009) showed that ACNs were detected in urine within 30 min after stomach intubation of black raspberry extract. Studies have also shown that the absorption of ACNs is increased with phytic acid (Matsumoto et al., 2007) and delayed with dairy cream (Mullen, Edwards, Serafini, & Crozier, 2008),

corroborating the involvement of the stomach with their absorption. Matsumoto et al. (2007) showed that the concomitant ingestion of ACNs and phytic acid reduced the GI transit time, whereas the dairy cream would be emptied at a slower rate from the stomach due to the fat content (Mullen et al., 2008).

However, the flavylium cation form has limited chances of being absorbed by passive diffusion (Lipinski et al., 1997), and evidence has suggested that bilitranslocase (TCDB 2.A.65.1.1) (Saier, 2000) is involved in the absorption of ACNs in the stomach (Passamonti et al., 2003, 2005a). Bilitranslocase is an ATP-independent uniporter carrier protein first identified in the liver (Sottocasa, Lunazzi, & Tiribelli, 1989), where its role is in assisting the hepatic detoxification process and transporting organic anions from the blood into the hepatocytes. This protein has also been identified in rodent intestine (Battiston, Macagno, Passamonti, Micali, & Sottocasa, 1999) and kidney (Elias et al., 1990), and the vascular endothelium of rats and humans (Maestro et al., 2010). It should be noted that under certain conditions, this carrier can reach saturation, i.e. increasing the concentration of ACNs has resulted in an observable reduction in the amount absorbed (Fernandes et al., 2012; Kurilich et al., 2005; Talavéra et al., 2003).

The ACNs that are not absorbed in the stomach are subject to extensive degradation by changes in intestinal pH (pH 7.5–8.0) (Liu et al., 2014; McDougall et al., 2005) and metabolism by intestinal microbiota (Fleschhut et al., 2006) and phase II enzymes (Czank et al., 2013; Ferrars et al., 2014; Fleschhut et al., 2006; Kay et al., 2005). As the pH is increased, the structure of ACN transforms into a pseudobase, quinoidal base, and then a chalcone, which is subject to nucleophilic attack by water (Brouillard & Delaporte, 1977; Brouillard & Dubois, 1977; Oliveira et al., 2006). These transformations would limit the amount of ACN glycosides that can reach the various tissues and sites in the body in their native form and, consequently, affect the translation of results from *in vitro* experiments into *in vivo* evidence.

Researchers have also shown that the administration of high concentrations of ACNs often do not result in a proportionally higher bioavailability and biological effect (Adisakwattana, Yibchok-Anun, Charoenlertkul, & Wongsasiripat, 2011; Banaszewski, Park, Edirisinghe, Cappozzo, & Burton-Freeman, 2013; Charron et al., 2009; Keane et al., 2015; Wei, 2014). For instance, Adisakwattana et al. (2011) investigated the effects of

three concentrations of cyanidin 3-rutinoside (30, 100, and 300 mg/kg) in lowering blood glucose concentration in Wistar rats in comparison to a control (distilled water), followed by the administration of a maltose or sucrose solution (3 g/kg). The highest concentrations (100 and 300 mg/kg) had a similar and significant effect on the AUC of glucose during the time of the experiment (180 min) in comparison to the control and prevented the increase in concentration potentially due to an inhibition of α-glucosidase (Adisakwattana et al., 2011). In healthy volunteers, Charron et al. (2009) showed that the ingestion of different amounts of purple carrot juice (50, 150, and 250 mL) resulted in similar AUC for 150- and 250-mL treatments, which could indicate that the absorption could have been saturated. Keane et al. (2015) also showed a similar AUC and maximum concentration of protocatechuic acid, an ACN metabolite (Ferrars et al., 2014; Vitaglione et al., 2007), after ingestion of 30 and 60 mL of Montmorency tart cherry concentrate by healthy volunteers.

In this context, GRS can be a promising delivery platform to modulate the release and absorption of ACNs for the management and control of T2D and for increased health benefits. The sustained release of ACN glycosides would also prevent the saturation of carriers (such as bilitranslocase) and reduce the concentrations needed to observe an effect *in vivo*. As it has been shown that the uptake of parent ACNs by bilitranslocase is rapid (Talavéra et al., 2003; Vanzo et al., 2011), the goal should be to control the release of ACNs over time instead of increasing the concentration. Previous research has shown, for example, that GRS can improve the absorption and bioavailability of drugs with significantly higher AUC in comparison to conventional delivery systems, such as determined for ofloxacin (Shakyaa, Thapa, & Saha, 2013), theophylline (Miyazaki, Yakou, & Takayama, 2008), and cefuroxime (Bomma & Veerabrahma, 2013).

This section provides an overview of the gastric motility and the different strategies that can be explored to increase the retention time of ACNs in the upper GI tract.

5.4.1 Gastric Motility and Emptying

The gastric motility pattern can be distinguished between fasted and fed states (Dooley, Di Lorenzo, & Valenzuela, 1992). Undigested particles from ingested food and mucus residue remain in the stomach until approximately 2 h after the digested food has left this organ (Read, Al-Janabi, Holgate, Barber, & Edwards, 1986). Migrating myoelectric complex (MMC) or *housekeeper contractions* are responsible for removing the residues left in the stomach after digestion through strong and cyclic contractions against an open pylorus. This cycle is divided into four phases based on the contraction strength and repeated every two hours until a meal is eaten and digestion starts (Code & Marlett, 1975).

The time it takes to empty the stomach depends on the physical state of the food (Olausson et al., 2008; Urbain et al., 1989; Vincent et al., 1995), its quantity (Hunt & Spurrell, 1951), composition (Gentilcore et al., 2006), and caloric content (Moore, Christian, & Coleman, 1981). The presence of food will induce a fed pattern of motility, with regular tonic and peristaltic contractions (de Wever, Eeckhout, Vantrappen, & Hellemans, 1978). At this stage, peristaltic waves will mix, grind, and empty the stomach simultaneously. Emptying is a critical step during digestion and occurs when the solids have been reduced to particles smaller than approximately 2 mm (Vincent et al., 1995). It has been suggested that particles up to 10 mm can be emptied from the stomach in the fed state in humans (Davis, 2005).

In relation to the food consistency, Clark et al. (1993) evaluated the effect of isotopically labeled solid, semisolid, and liquid meals on gastric pH and emptying time. They reported that the rate of gastric emptying was higher for liquid and semisolid meals, varying from 9.8–103.3 min (mean 35.6 min) and 33.5–120 min (mean 47.4 min), respectively, while for solid meals the range was 45.0–103.8 min (mean 72.0 min) (Clark et al., 1993). Liquid emptying occurs as first-order kinetics, with emptying being directly proportional to the volume present in the stomach (Hunt & Spurrell, 1951). In the case of solids, studies have shown a biphasic pattern: little emptying occurs initially (lag phase, possibly the time required to reduce the particle size), followed by linear emptying (independent of gastric volume) (Fallatah et al., 2013).

In relation to the composition and caloric density, approximately 2–4 kcal are delivered to the duodenum by the stomach per minute. Meals with similar energy content are emptied from the stomach at similar rates, and this rate is unlikely to be affected by consistency or composition (Faas et al., 2002). It has also been suggested that food could form layers in the body of the stomach when an upright position is adopted and if the meal contains a sufficient amount of fat (Wiggins & Dawson, 1961) – this would be due to density differences and weak peristaltic contractions, which would separate fat from water. Another explanation would be the lower rate of fat absorption in the intestine (Lin, Zhao, & Wang, 1996).

The rate of gastric emptying is also influenced by biological factors, such as body mass index (Brogna et al., 1998; Jackson et al., 2004), hormonal factors (Hutson, Roehrkasse, & Wald, 1989), gender (Lorena et al., 2000), glycaemia (Woerle et al., 2008), posture (Steingoetter et al., 2006), stress (Mistiaen, Blockx, Van Hee, Bortier, & Harrisson, 2002; Ochi et al., 2008), and pathological states (Buckles, Sarosiek, McMillin, & McCallum, 2004; Hardoff et al., 2001). For instance, Hardoff et al. (2001) showed that the emptying time is delayed in patients with Parkinson's disease in comparison with healthy volunteers.

5.4.2 Classes of Gastroretentive Systems

GRS differ from conventional delivery systems because they remain in the stomach for a longer period of time, prolonging the residence time of the bioactive in the upper GI tract (Joseph et al., 2002). Longer gastric retention can be achieved by different strategies, such as mucoadhesion (Md et al., 2011), flotation (Ichikawa, Kato, Kawahara, Watanabe, & Kayano, 1991), high density systems (Bechgaard & Ladefoged, 1978), and expansion (Urquhart & Theeuwes, 1984). An overview of these different classes of GRS, highlighting the ones that are interesting for food applications, is presented in Figure 5.1. This section will review different strategies that can be used for the development of GRS that may be of interest for the food industry, based on information described in the pharmaceutical literature.

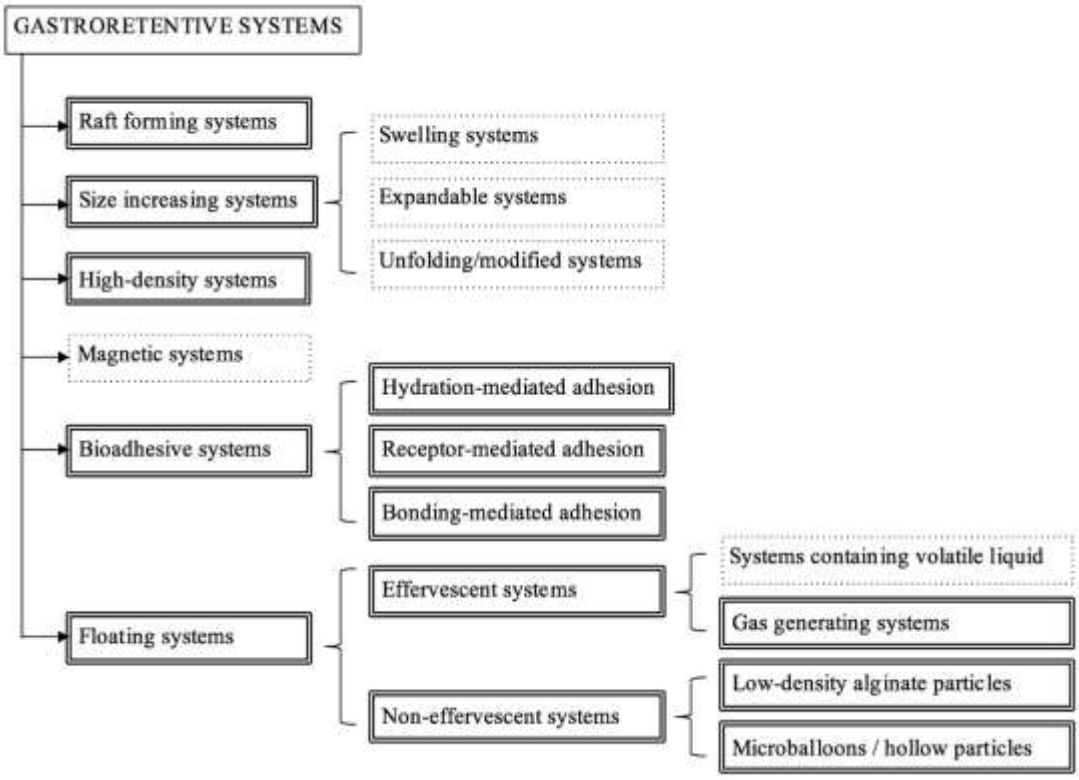

Figure 5.1 Overview of different GRS. GRS applicable for food applications are highlighted with double lines. The other approaches (dotted lines) will not be discussed in this review as they may involve the use of non-GRAS solvents or polymers and be less suitable for the food industry

Raft-Forming Systems. These systems consist of liquid hydrogels (e.g. alginates) that gel when in contact with body fluids or due to changes in pH (Kubo, Konno, Miyazaki, & Attwood, 2004), forming a continuous layer that is termed *raft*. The systems are often formulated with gas-generating compounds (e.g. carbonate salts) that release CO_2 gas in the acidic conditions of the stomach. The gas is entrapped in the gel matrix, making the system less dense and contributing to its buoyancy in relation to the gastric content (Tang, Alvani, & Tester, 2010), allowing it to float. For example, Ibrahim (2009) showed that a system consisting of sodium alginate and a gas-generating compound (release of CO_2 gas for buoyancy) gelled instantaneously once in contact with simulated gastric fluid (0.1 N HCl) and remained intact and buoyant for more than 48 h.

High-Density (or Sinking) Systems. Hoelzel (1930) provided the first evidence that the GI transit time could be prolonged by increasing the density of a dosage form. These

systems have a density higher than the gastric fluids, which is designed to delay gastric emptying (Clark et al., 1993). Clarke, Newton, and Short (1995) proposed that the critical density to observe a prolonged residence time in the stomach is between 2.4 and 2.8 g cm^{-3}. Devereux, Newton, and Short (1990) showed that pellets with density of 2.8 g cm^{-3} stayed longer in the stomach that was either in fed and fasted states than pellets with 1.5 g cm^{-3} density. The density of the GRS can be increased by coating it with a heavy inert material (e.g. zinc oxide) (Singh & Kim, 2000); however, the use of high-density GRS as a platform to increase the bioavailability of certain compounds has been questioned (Rouge et al., 1998).

Bioadhesive Systems. In the context of this review, bioadhesion refers to the binding of a natural or synthetic polymer (in the form of a delivery system) to a biological substrate, such as a mucous layer (hence the term mucoadhesion) (Henriksen, Green, Smart, Smistad, & Karlsen, 1996). Different theories proposed to explain the adhesion process have been reviewed (Boddupalli, Mohammed, Nath, & Banji, 2010; Shaikh, Singh, Garland, Woolfson, & Donnelly, 2011).

Low-Density (or Floating) Systems. One of the first descriptions of floating systems was made by Davis (1968), who employed pills with density below 1.0 g cm^{-3} to overcome issues reported by patients when swallowing medicinal pills. However, it has also been reported that sufficient liquid, such as a glassful of water (200–250 mL), is necessary to facilitate the floatability of these systems (Soppimath, Kulkarni, Rudzinski, & Aminabhavi, 2001). The presence of liquid will result in emptying the stomach following a first-order process with half-time of ~30–50 min. If no liquid is ingested during this period, after 2 h there will be insufficient fluid remaining in the stomach on which the unit can float (Davis et al., 1986), which would result in the elimination of the GRS.

To promote buoyancy, both effervescent and non-effervescent systems have been used. Effervescent FDS combine swellable polymers, such as chitosan, and effervescent compounds (sodium bicarbonate and citric or tartaric acids). For instance, the optimal stoichiometric ratio of citric acid and sodium bicarbonate has been determined as 0.76:1

(Saxena, Gaur, Singh, Sing, & Dashora, 2014). Another approach is the use of resin beads loaded with the gas-generating agent, which is later coated with ethyl cellulose. Since this polymer is insoluble but permeable, it will allow the permeation of water and entrapment of carbon dioxide gas (Saxena et al., 2014).

Non-effervescent FDS are based on gel-forming or swellable polymers, such as hydrocolloids, polysaccharides, and matrix-forming compounds (e.g. polycarbonate). It is expected that these polymers will swell once in contact with gastric pH and trap gas within their structure, maintaining their integrity (Singh & Kim, 2000).

Although GRS could potentially improve the delivery of ACNs for the management of diseases, such as T2D, more research is needed to determine the most appropriate GRS platforms for the delivery of ACNs and their implications on degenerative diseases.

5.5 CONCLUSIONS

There is evidence that ACNs could potentially be used to effectively manage and control degenerative diseases such as T2D (Hoggard et al., 2013; Muraki et al., 2013; Samieri et al., 2014; Stull et al., 2010; Törrönen et al., 2010, 2012a, 2012b; Wedick et al., 2012). GRS could potentially deliver and maintain ACN glycosides obtained from plant sources in the upper GI tract where their stability and absorption are favoured. As discussed in this review, there are different categories of GRS that may be applicable to the food industry, using materials that are GRAS. More research is needed in the formulation and characterization of the best GRS to be used for the administration of ACNs for T2D and other degenerative diseases. In addition, GRS may be a suitable vehicle for other food-derived extracts that are rich in bioactive compounds, to increase bioavailability and maximize the potential health benefits, and potentially reduce the current gap when translating *in vitro* effects into clinical responses.

Chapter 6 OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF ANTHOCYANINS FROM HASKAP BERRIES (*LONICERA CAERULEA* L.) USING RESPONSE SURFACE METHODOLOGY

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Celli, G.B., Ghanem, A., & Brooks, M.S. (2015). Optimization of ultrasound-assisted extraction of anthocyanins from haskap berries (*Lonicera caerulea* L.) using Response Surface Methodology. *Ultrasonics Sonochemistry*, *27*, 449–455.

6.1 ABSTRACT

Haskap berries (*Lonicera caerulea* L.) are a rich source of bioactive molecules. As such, the extraction of anthocyanins (ACNs) is important for the development of many value-added products and functional food ingredients. In this paper, the ultrasound-assisted extraction (UAE) of ACNs from haskap berries was investigated. Significant independent variables were screened and optimized using Plackett–Burman (PB) and Box–Behnken (BB) designs, respectively. The mathematical model showed a high coefficient of determination (R^2 = 0.9396) and the optimum conditions for the extraction were as follows: liquid:solid ratio of 25:1 (mL/g), solvent composition of 80 % ethanol, addition of 0.5 % formic acid, and ultrasound bath temperature of 35 °C for 20 min. Under these conditions, the total ACN content of 22.73 mg cyanidin 3-glucoside equivalents (C3G)/g dry weight (DW) was consistent with the predicted response of 22.45 mg C3G/g DW from the model (mean error of 1.28 %). Five ACNs were identified in the optimized extract, namely cyanidin 3,5-diglucoside, cyanidin 3-glucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside, and peonidin 3-glucoside. Thus, UAE is a suitable technique for the extraction of ACNs from haskap berries.

6.2 INTRODUCTION

It is now well-accepted that fruits and vegetables are an essential part of our diet and their regular consumption is associated with health benefits, including the reduced

incidence of degenerative disorders (Beattie et al., 2005). In this context, it has been demonstrated that anthocyanins (ACNs) contribute significantly to the antioxidant activity and health-promoting effects exhibited by fruits and their products (D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010). Besides their use as food colorants, these compounds have been associated with an improvement of blood glucose levels by inhibiting α-glucosidase (Adisakwattana, Charoenlertkul, & Yibchok-Anun, 2009), promoting insulin secretion (Jayaprakasam et al., 2005) and improving sensitivity to insulin (Nizamutdinova et al., 2009; Seymour et al., 2009), which can help with the prevention and management of diabetes. Jennings et al. (2014) showed in a cross-sectional study with 5,119 healthy women that the intake of ACNs was positively associated with a reduction of blood insulin levels due to an improvement in insulin resistance.

In order to recover, identify, and quantify these compounds in food matrices, various techniques have been developed to increase the efficiency of liquid extractions, decrease the processing time, and minimize the use and exposure to organic solvents. Examples of techniques are supercritical fluid extraction, pulsed electric fields, pressurized liquid extraction, microwave- and ultrasound-assisted extraction (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2010; Stalikas, 2010; Tsao & Deng, 2004). For instance, the use of ultrasound permits an efficient extraction of bioactive molecules in a shorter time and at lower temperatures than traditional methods such as maceration. This can be attributed to the cavitation phenomenon that occurs as a result of the propagation of ultrasound pressure waves. The eventual collapse of cavitation bubbles, in combination with highly localized temperatures, leads to the disruption of cell walls and, consequently, the release of cellular content to the extraction medium (Ebringerová & Hromádková, 2010).

Researchers have previously used ultrasound-assisted extraction (UAE) to obtain phytochemicals from haskap berries (*Lonicera caerulea* L.) (Bakowska-Barczak et al., 2007; Chaovanalikit et al., 2004; Kusznierewicz et al., 2012; Skupień et al., 2009; Zhao et al. 2011, 2012). As haskap berries have particularly high levels of phytochemicals, mainly in the form of vitamin C and polyphenolic compounds, they have attracted much interest upon their recent introduction to North America (Bors et al., 2012). The Japanese

Ainu aboriginals have traditionally referred to these fruits as the *elixir of life* (Thompson, 2006) and the health benefits associated with these berries have been the subject of a recent review (Celli et al., 2014).

To the best of our knowledge, none of the published studies on the UAE of ACNs from haskap berries report on optimization of conditions. Thus, the objective of the present study was to establish the optimal UAE conditions for the extraction of ACNs from haskap berries. Statistical optimization was carried out by: (a) screening the most significant variables affecting the extraction using Plackett–Burman design; (b) optimization of the significant variables by Response Surface Methodology using Box–Behnken design; and (c) experimental validation of the model with repeated measurements under the optimized conditions and comparison to the predicted value. During screening, total ACN content (TAC) measurements were also analyzed to determine if there was any correlation with total phenolic content (TPC). Then, the ACN profile of the optimized extract was determined by HPLC.

6.3 MATERIAL AND METHODS

6.3.1 Plant Material

Fully-ripe haskap berries (variety *Indigo Gem*) were used in this study. Berries were mechanically harvested at the Northern Light Orchards (Saskatchewan, Canada), packed, frozen, and shipped to LaHave Natural Farms, in Nova Scotia. The berries were then transferred to Dalhousie University (Department of Process Engineering and Applied Science) and kept at −35 °C prior to freeze drying. Frozen berries were freeze-dried for approximately three days in a Labconco FreeZone 4.5 L Bench-top Freeze Dry System (Labconco, Kansas City, MO, USA) until the final moisture content was below 5 % (w/w). The freeze-dried samples were stored in a desiccator at −18 °C until analysis. Freeze-dried haskap berries were then ground in a domestic grinder (Everyday Essentials model E710) and passed through a 0.5 mm-sized sieve prior to extraction.

6.3.2 Chemicals and Reagents

Folin–Ciocalteu phenol reagent 2 N, formic acid, and reagent ethanol were purchased from Fisher Scientific (Ottawa, ON, Canada). HPLC grade methanol and standards were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

6.3.3 Equipment

The extraction procedure was conducted in an ultrasound bath Branson 2510R-DTH (Bransonic, Danbury, CT, USA), with fixed frequency (40 kHz) and power (100 W). The temperature was controlled within ± 0.5 °C with a calibrated thermometer and adjusted with cold water.

6.3.4 Experimental Design and Data Analysis

Optimization of the UAE of ACNs from haskap berries was performed in two stages. The first stage consisted of the screening and identification of variables (or factors) that have significant effect on the extraction. In the second phase, the optimum values of these variables for the UAE were determined.

In both stages, ground haskap berry samples were transferred to 25 mL glass tubes with screw caps and the extraction procedure was conducted, according to the experimental design conditions under study. After the extraction, the contents were centrifuged using a Sorvall RT1 Centrifuge using a T41 Swing-Out Rotor (Osterode, NI, Germany) at 2232 × *g* for 20 min at 4 °C. The supernatant was removed and filtered through a 0.45-μm syringe filter into capped tubes wrapped in aluminum foil. The samples were immediately analyzed in triplicate for TAC and TPC. TAC measurements were used for screening and optimization, while TPC measurements were used as a comparison with TAC measurements during screening only. TPC was also tested during screening stage to assess its relationship with TAC and this was reported as the Pearson's correlation coefficient (*r*).

The following sections describe the strategies used for screening and optimization of variables that affect the UAE of ACNs from haskap berries.

6.3.4.1 Plackett–Burman (PB) Design for Screening of Variables

The Plackett–Burman experimental design was used in this study to identify the variables that significantly affect the extraction of phenolic compounds, such as ACNs (Plackett & Burman, 1946). Based on the studies that reported the use of UAE from haskap berries (Bakowska-Barczak et al., 2007; Chaovanalikit et al., 2004; Kusznierewicz et al., 2012; Skupien et al., 2009; Zhao et al. 2011, 2012), the proportion of solvent to ground haskap (ξ_1), ethanol concentration (ξ_2), formic acid concentration (ξ_3), ultrasound (US) bath temperature (ξ_4), and extraction time (ξ_5) were considered as input variables in the experimental design and further investigated. Each variable was coded as x_1 to x_5 and examined in two levels, namely –1 (low level) and +1 (high level) (Table 6.1). PB design assumes that the main effects of the variables have no interactions and is based on a first-order polynomial model, as follows:

$$y = \beta_0 + \sum_{i=1}^{5} \beta_i x_i \tag{25}$$

where y is the response; β_0 is the constant (or model intercept); β_i is the linear regression coefficient; and x_i is the level of the independent variable under study.

Table 6.1 Input variables (natural and coded) and levels chosen for screening

Input variable	Coded variable	Levels	
		–1	+1
Proportion solvent:solid (mL/g) (ξ_1)	$x_1 = (\xi_1 - 15)/10$	5:1	25:1
Ethanol concentration (%) (ξ_2)	$x_2 = (\xi_2 - 85)/15$	70	100
Formic acid concentration (%) (ξ_3)	$x_3 = (\xi_3 - 0.5)/0.5$	0	1
US bath temperature (ºC) (ξ_4)	$x_4 = (\xi_4 - 35)/10$	25	45
Extraction time (min) (ξ_5)	$x_5 = (\xi_5 - 20)/10$	10	30

The variables were screened in 15 runs, with the center point in triplicate, according to the design matrix presented in Table 6.2, and the response was measured as TAC. Center points were included to detect any curvature or deviation in linearity that may exist in the model. Variables that were significant at $p < 0.05$ in the regression analysis were considered to have an impact on the response and selected for optimization. Minitab® version 17.1.0 software (Minitab Inc., PA, USA) was used for the experimental design and statistical analysis.

Table 6.2 Plackett–Burman design matrix with coded variables, including three center points, and experimental results for TAC

Run No.[a]	x_1	x_2	x_3	x_4	x_5	y = **TAC (mg/g)**
1	+1	–1	+1	–1	–1	21.23
2	+1	+1	–1	+1	–1	13.65
3	–1	+1	+1	–1	+1	8.36
4	+1	–1	+1	+1	–1	20.36
5	+1	+1	–1	+1	+1	18.02
6	+1	+1	+1	–1	+1	15.77
7	–1	+1	+1	+1	–1	8.75
8	–1	–1	+1	+1	+1	13.81
9	–1	–1	–1	+1	+1	14.18
10	+1	–1	–1	–1	+1	21.92
11	–1	+1	–1	–1	–1	6.63
12	–1	–1	–1	–1	–1	11.23
13	0	0	0	0	0	19.82
14	0	0	0	0	0	19.02
15	0	0	0	0	0	19.14

Note: [a] Run number was used for identification purposes only and does not indicate the order in which the experimental runs were conducted

The main effect of each variable was calculated by:

$$Effect_{x_i} = (2/N)[\sum x_i\,(+1) - \sum x_i(-1)] \quad (26)$$

where N is the total number of experiments (center points were not considered); x_i (+1) and x_i (–1) terms are the responses when a given factor is at its high and low levels, respectively. When calculated by this formula, main effects of each factors are not confounded with other effects, though their interactions would be confounded.

6.3.4.2 Box–Behnken (BB) Design for Optimization of UAE Conditions

The BB design is a type of Response Surface Methodology (RSM) (Montgomery, 2013) and was used to optimize the variables and their interactions that were found to be significant in the extraction of ACNs from haskap berries. For BB design, three levels were considered: –1, 0, and +1, representing low, middle, and high levels, respectively. Similar to the PB design, a total of 15 runs (with the center point in triplicate) were used to optimize the significant variables previously determined during screening stage, namely liquid:solid ratio (ξ_1), solvent concentration (ξ_2), and extraction time (ξ_5). The design matrix and experimental results are presented in Table 6.3. Design-Expert® version 9.0 software (Stat-Ease, Inc., Minneapolis, MN, USA) was used for constructing the design matrix, graph plotting, and data analysis. Results using response surface regression were fitted to a second-degree polynomial equation, as follows:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i\, x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_i \sum_{j=i+1} \beta_{ij} x_i x_j \quad (27)$$

where y is the response; β_0 is the constant (or model intercept); β_i is the linear coefficient of the coded variables; β_{ii} is the quadratic coefficient; β_{ij} the cross-product coefficient; x_i and x_j are the coded independent variables. Contour and three-dimensional surface plots were used to demonstrate the influence of coded variables in the response. The experimental validity of the model was determined by comparison of practical results and predicted values at the optimal conditions.

Table 6.3 Box–Behnken design matrix and experimental results

Run No.[a]	x_1	x_2	x_5	y = TAC (mg/g)
1	–1	–1	0	14.37
2	+1	–1	0	21.08
3	–1	+1	0	8.75
4	+1	+1	0	17.36
5	–1	0	–1	12.57
6	+1	0	–1	22.52
7	–1	0	+1	13.37
8	+1	0	+1	22.06
9	0	–1	–1	13.54
10	0	+1	–1	9.92
11	0	–1	+1	16.06
12	0	+1	+1	13.05
13	0	0	0	16.64
14	0	0	0	15.84
15	0	0	0	16.51

Note: [a] Run number was used for identification purposes only and does not indicate the order in which the experimental runs were conducted

6.3.5 Determination of Total Anthocyanin Content (TAC)

The TAC of the extracts was determined by the pH-differential method described by Giusti and Wrolstad (2001). Absorbances were measured at λ_{max} (determined as 515 nm) and 700 nm against a blank consisting of distilled water. Results were expressed as mg of cyanidin 3-glucoside equivalents (C3G) per g of berries (on a dry weight basis).

6.3.6 Determination of Total Phenolic Content (TPC)

The TPC of the haskap berry extracts was determined by the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999), with slight modification. Briefly, 500 μL of diluted extract (1:10) were added to 2.5 mL of Folin-Ciocalteu reagent freshly diluted to 0.2 N. After 5 min, 2 mL of sodium carbonate solution (75 g/L) was added to neutralize the mixture. The mixtures were left to react in the dark, at room temperature, for 120 min, after which the absorbance was measured at 760 nm in a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Madison, WI, USA). Results were expressed as mg of gallic acid equivalents (GAE) per g of berries (on a dry weight basis).

6.3.7 Determination of Anthocyanin Profile in the Optimized Extract by HPLC

The extract obtained at the optimal conditions was analyzed by HPLC for the determination of the ACN profile. The chromatographic separation was carried out on a high performance liquid chromatographer Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD) and autosampler. The column used was a 250 × 4.6 mm i.d., 4 μm, Synergi Max-RP C12 (Phenomenex, Torrance, CA, USA) with a universal SecurityGuard™ KJ0-4282 system (Phenomenex). The samples (20 μL) were eluted through the column with a gradient mobile phase consisting of A (10 % (v/v) methanol acidified with 1 % (v/v) formic acid in water) and B (0.1 % (v/v) formic acid in methanol) with a flow rate of 0.8 mL/min. A 40-min linear gradient was programmed as follows: 0–7 min, 10–20 % B; 7–20 min, 20–45 % B; 20–25 min, 45–70 % B; 25–28 min, 70–100 % B; 28–31 min, 100 % B; 31–40 min, 100–10 % B. The column temperature was set to 30 °C. The chromatograms were acquired at 520 nm and the peaks were identified through the comparison of their retention time and absorbance spectra to that of commercial standards.

6.4 RESULTS AND DISCUSSION

6.4.1 PB Design for Screening of Variables

A PB design consisting of 15 runs (including three center points) was employed to assess the effects of selected independent variables on the UAE yield of ACNs from haskap berries. This screening design was previously used to determine the significant variables that affect the extraction of phenolic compounds from grapes (Dopico-García, Valentão, Guerra, Andrade, & Seabra, 2007) and hawthorn seeds (Pan, Yu, Zhu, & Qiao, 2012). Low and high values for each factor were determined based on studies using haskap berries described in the literature (Bakowska-Barczak et al., 2007; Chaovanalikit et al., 2004; Kusznierewicz et al., 2012; Skupien et al., 2009; Zhao et al. 2011, 2012). Ethanol was the solvent of choice for the extraction, as previous studies indicate that aqueous solutions of ethanol were as good or more efficient than methanol for the extraction of anthocyanins from haskap berries (Myjavcová et al., 2010), red currant, and grapes variety Pinot Noir (Lapornik, Prošek, & Wondra, 2005) and cultivar Benitaka (Vanini, Hirata, Kwiatkowski, & Clement, 2009). Added advantages of ethanol are its reduced toxicity and acceptability in food applications.

Among the variables under investigation, the proportion of liquid:solid (x_1), solvent concentration (x_2), and extraction time (x_5) were significant for TAC measured by spectrophotometric method (Table 6.4). The proportion liquid:solid (x_1) and extraction time (x_5) exerted a positive effect, whereas solvent concentration (x_2) had a negative impact on ACN yield. Ivanovic et al. (2014) also identified sonication time as a variable that positively affects the TAC recovery from blackberries.

Table 6.4 Main effect of each input variable on TAC

Input variable	Main effect
Proportion of liquid to haskap powder (x_1)	8.00*
Ethanol concentration (x_2)	-5.26*
Formic acid concentration (x_3)	0.44
US bath temperature (x_4)	0.60
Extraction time (x_5)	1.70*

* significant at $p < 0.05$

Based on the analysis of variance presented in Table 6.5, it is apparent that the model for TAC was highly significant ($p < 0.0005$). Also, the lack-of-fit was not significant, which implies that the model fits the experimental data well. Small values of the standard error of the regression (S) at $p < 0.05$ indicated that the observations were close to the fitted line, while 97.09 % of the variance about the mean for TAC was explained by the fitted model (represented by regression coefficient, R^2). The linear equations (in coded and natural variables) obtained for PB design of TAC were, respectively:

$$y_{TAC} = 14.49 + 4.00x_1 - 2.63x_2 + 0.85x_5 \quad \text{(coded variable)} \quad (28)$$

$$y_{TAC} = 21.70 + 0.40\xi_1 - 0.17\xi_2 + 0.09\xi_5 \quad \text{(natural variable)} \quad (29)$$

The analysis also revealed that the curvature was highly significant ($p < 0.0005$), indicating that at least one variable was involved in an order higher than one. The optimum region would be near or within the experimental ranges selected for the screening design and the steepest ascent method was not performed (Montgomery, 2013). For this reason, the linear model would not be appropriate for determining the optimum conditions of the significant variables. Box–Behnken design was performed to obtain a higher order model and further optimization of the variables.

It has been previously reported that the TAC content of haskap berries of different cultivars other than *Indigo Gem* can range between 116 and 1,400 mg of C3G per 100 g of fresh weight (Bakowsja-Baczak et al., 2007; Deineka et al., 2005; Fan et al., 2011;

Lefèvre et al., 2011; Thompson and Chaovanalikit, 2003). This wide range could be attributed to the different extraction conditions used and the natural variability between cultivars and locations of harvesting. The values obtained in this study under different experimental conditions range from 99 to 329 mg C3G/100 g fresh weight (FW), which is slightly below the lower limit described by other authors.

Rupasinghe et al. (2012) evaluated the TPC and antioxidant activity of haskap berries of different cultivars, including *Indigo Gem*. Even though these authors did not report on the results for TAC, the TPC values can be compared to those obtained in this study. The reported values for TPC for fruits from this variety were 500.78 ± 48.5 mg GAE/100 g FW after extraction using methanol at room temperature, in a 5:1 ratio of solvent:frozen berries. Our TPC results, converted to a fresh weight basis, would lead to a range of 107.93–527.50 mg GAE/100 g FW, depending on the extraction conditions. The maximum TPC measurement from our optimized extraction conditions indicates that near-optimal extraction conditions were achieved in the other study, although the authors did not report on the extraction time, which we demonstrated to be a significant factor during screening of variables affecting the UAE of anthocyanins. These values also indicate that UAE of ACNs using ethanol as the solvent is comparable to maceration using methanol. However, it should be noted that some variability in the ACN content of the fruits would be expected, due to differences in the growing, harvesting, and storage conditions.

The Pearson's correlation coefficient (r) was calculated for TPC and TAC prior to RSM analysis in order to assess the contribution of ACNs to the phenolic content. Some authors have reported a good correlation between TPC and other antioxidant capacity tests based on reduction efficiency (Huang et al., 2005), and the contribution of ACNs to the overall antioxidant activity of a fruit matrix has been well documented (Rivero-Pérez, Muñiz, & González-Sanjosé, 2008). In the present study, a highly positive relationship ($r = 0.987$, $p < 0.0005$) was found for TAC and TPC for the screening stage. These results indicate that ACNs, as expected, could act as reducing agents in the Folin–Ciocalteu assay for TPC. The Folin–Ciocalteu assay does lack in specificity (Huang et al., 2005) and the results could also be overestimated by interference with ascorbate, for example, which can be found in high concentrations in haskap berries (Jurikova et al., 2012). In

this work no correction was made in the FC assay for potential interference with vitamin C in the haskap berries. The pH-differential method for TAC, on the other hand, has good specificity and therefore was used in this study for the optimization of the parameters that significantly affected the extraction of ACNs.

6.4.2 RSM for the Optimization of UAE Conditions

RSM was used in this study for the statistical optimization of UAE of ACNs from haskap berries. This design has been used previously for mulberry (T. Zou et al., 2011), Chinese sumac (Lai et al., 2014), and litchi (Rao, 2010). The ANOVA results showing the significant variables for UAE of ACNs from haskap berries analyzed by RSM are presented in Table 6.5.

As observed in the ANOVA table, the quadratic model containing only significant terms was significant ($F = 38.87$, $p < 0.0001$). It also indicated that x_1, x_2, x^2_1, and x^2_2 were significant terms in the model ($p < 0.05$), and that this model explained 93.96 % of the variation in TAC, leaving approximately 6 % to be explained by noise. The coefficient of variation (CV% = 7.65) could also be used as an indication of the precision and reliability of the model. As a result, the quadratic model in coded and natural variables were:

$$y_{TAC} = 16.00 + 4.31x_1 - 1.99x_2 + 1.75x_1^2 - 2.60x_2^2 \quad \text{(coded variables)} \quad (30)$$

$$y_{TAC} = -58.85 - 0.09\xi_1 + 1.83\xi_2 + 0.02\xi_1^2 - 0.01\xi_2^2 \quad \text{(natural variables)} \quad (31)$$

The response surface graph of TAC in relation to solvent:solid ratio and solvent concentration is illustrated in Figure 6.1, with time kept at its level 0 (20 min). An increase in the proportion of solvent to dried haskap sample resulted in higher ACN extraction and reached a maximum at 25:1, which could indicate a saturation of the extraction media. At the highest level of liquid:solid ratio, higher values were obtained with solvent concentration slightly below the center point, and there was a reduction of the predicted TAC response with 100 % ethanol.

Table 6.5 ANOVA results of screening and optimization stages using TAC as response

Source	*df*	SS	MS	*F*-value	*p*-value
SCREENING					
Model	4	339.63	84.91	83.37	<0.0005
Linear	3	283.58	94.53	92.82	<0.0005
x_1	1	191.87	191.87	188.40	<0.0005
x_2	1	83.02	83.02	81.52	<0.0005
x_5	1	8.69	8.69	8.53	0.015
Curvature	1	56.05	56.05	55.04	<0.0005
Error	10	10.18	1.018		
Lack-of-fit	8	9.81	1.23	6.61	0.138
Pure error	2	0.37	0.19		
Total	14	349.81			
S	1.01		R^2 (adjusted)	0.9592	
R^2	0.9709		R^2 (predicted)	0.9345	
OPTIMIZATION					
Model	4	219.97	54.99	38.87	<0.0001
x_1	1	148.95	148.95	105.27	<0.0001
x_2	1	31.81	31.81	22.48	0.0008
x_1^2	1	11.40	11.40	8.06	0.0176
x_2^2	1	25.19	25.19	17.80	0.0018
Error	10	14.15	1.41		
Lack-of-fit	8	13.78	1.72	9.33	0.1004
Pure error	2	0.37	0.18		
Total	14	234.12			
S	1.19		R^2 (adjusted)	0.9154	
R^2	0.9396		R^2 (predicted)	0.8602	

Note: *S* – standard error of the regression; R^2 – regression coefficient

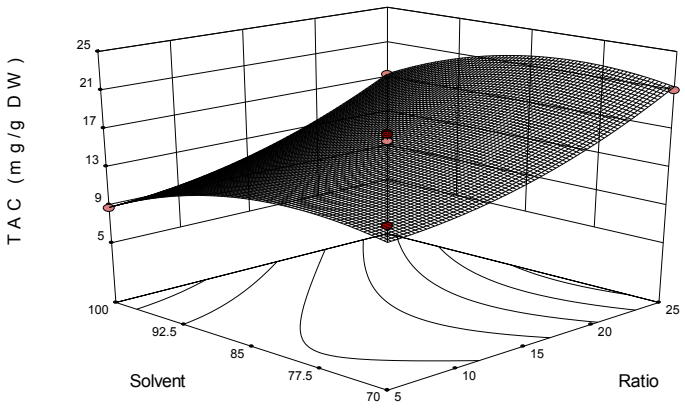

Figure 6.1 Response surface showing a smooth saddle-shaped graph plotting the response (TAC) versus liquid:solid ratio (ξ_1) and solvent concentration (ξ_2) from haskap berries, with time set to 20 min

The maximum predictive value of TAC and the natural value of each variable were determined using Design-Expert®, in agreement with the plot analysis. The optimal conditions were listed as follows: liquid:solid ratio of 25:1 (mL/g), solvent composition of 80 % ethanol, addition of 0.5 % formic acid, US bath temperature of 35 °C, and 20 min of extraction time. Under these conditions, the predicted TAC was calculated as 22.45 mg C3G/g DW of haskap berries. Further experimental trials were performed in the optimal region to validate the model and compare the predicted response with the practical results. A mean value of 22.73 ± 0.8 mg C3G/g DW (n = 6) obtained in these experiments was within the 95 % confidence interval (mean error of 1.28 %) and validated the RSM model.

To our knowledge, this is the first description of the screening and optimization of UAE of ACNs from haskap berries, although this technique has been widely used for the extraction of bioactive compounds from these fruits. After the optimal conditions were established, the chemical composition of the optimized extracts was determined and will be described in more detail in the following section.

6.4.3 Effects of Ultrasound on Anthocyanin Recovery

ACNs are secondary metabolites mainly found in the epidermal and hypodermal cell layers of fruits during ripening. It is suggested that the intermediates of ACN

biosynthesis are produced in the cytosol, transported into the tonoplast for completion, and accumulation in the vacuoles. However, the exact location and constitution of these cell structures are not completely elucidated and it seems to vary among fruits (Bae, Kim, Kim, & Lee, 2006).

The use of ultrasound for the extraction of plant compounds such as ACNs is reported as an efficient technique for their recovery as it disrupts cell walls, enhancing mass transport (Chen et al., 2007; D'Alessandro, Dimitrov, Vauchel, & Nikov, 2014). This efficiency is associated with the formation of cavitation bubbles that create voids in the medium during compression and rarefaction cycles, which can exceed the critical molecular distance of the liquid. As a consequence, the compression of these bubbles releases energy, increasing the temperature and pressure locally. The combination of high temperature and pressure creates turbulence and hotspots where chemical and physical reactions are dramatically augmented, including the disruption of cell wall (Chemat, Huma, & Khan, 2011). As a consequence, the plant cell properties will be altered favoring the extraction of bioactive compounds (Barba, Brianceau, Turk, Boussetta, & Vorobiev, 2015a). Ultrasound could also be used as a pre-treatment to improve maceration using water as an environmentally-friendly method to recover ACNs (Barba, Galanakis, Esteve, Frigola, & Vorobiev, 2015b) and its efficiency requires further investigation using haskap berries.

Barba et al. (2015a) recently reported that UAE resulted in up to 34.9 % ACN/phenolic ratio during the extraction of ACNs from fermented grape pomace. Our results during the screening stage (62.5 to 92.19 %) were considerably higher than that previously reported. One potential explanation could be the considerably higher concentration of ACNs in haskap berries in comparison to fermented grape pomace. It is quite common to find ACNs weakly associated with other metabolites, such as phenolic compounds, in a process termed intermolecular co-pigmentation. This is a unique feature of the ACNs and serves to protect the core structure from nucleophilic attack and, consequently, stabilize the color (Delgado-Vargas et al., 2000). If this were the case in haskap berries, the extraction of ACNs would eventually lead to the recovery of phenolic compounds. As mentioned previously, another important consideration is that the TPC method is not specific (Huang et al., 2005). The high values obtained for TPC could

result from interference of other reducing substances, such as ascorbic acid, which is found in high concentrations in haskap berries.

6.4.4 Anthocyanin Profile of the Optimized Extract

ACNs are important water-soluble pigments in plants (Bueno et al., 2012). Haskap berries have garnered the interest of the scientific community due to their high levels of bioactive compounds, which are mainly in the form of ACNs (Bors et al., 2012). The characterization and determination of the ACN profile of the haskap berry extract prepared at the optimal conditions was performed by HPLC with comparison with standards. Figure 6.2 shows the HPLC chromatogram (at 520 nm) of the extract, where the peaks were numbered according to their retention time. Five ACNs were identified, namely cyanidin 3,5-diglucoside (1.25 mg/g DW), cyanidin 3-glucoside (16.75 mg/g DW), cyanidin 3-rutinoside (0.65 mg/g DW), pelargonidin 3-glucoside (0.29 mg/g DW), and peonidin 3-glucoside (0.58 mg/g DW), in accordance to other reports in the literature (Bakowska-Baczak et al., 2007; Chaovanalikit et al., 2004; Deineka et al., 2005; Gazdik et al., 2008b; Jordheim et al., 2007; Kusznierewicz et al., 2012; Ochmian et al., 2009; Svarcova, Heinrich, & Valentova, 2007). Besides the use as natural colorant, the ACNs extracted from haskap berries could be used for the development of health-promoting products. The total ACN content determined by HPLC is in good agreement with the pH-differential method (19.51 mg/g DW).

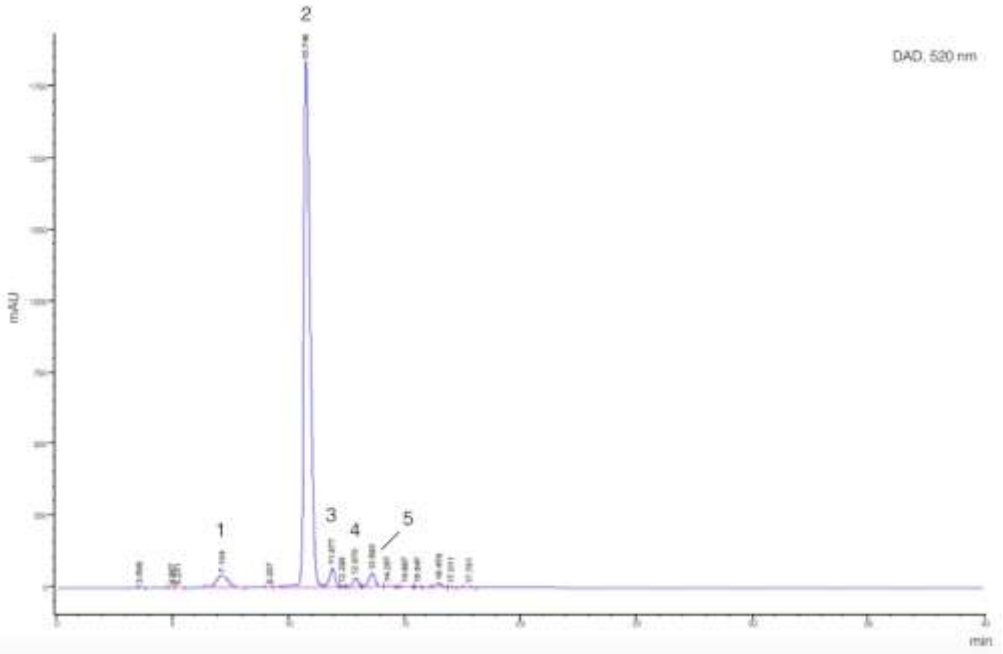

Figure 6.2 Chromatogram (0–40 min) recorded at 520 nm for extract prepared at the optimal conditions. Compounds were identified as follows: (1) cyanidin 3,5-diglucoside; (2) cyanidin 3-glucoside; (3) cyanidin 3-rutinoside; (4) pelargonidin 3-glucoside; and (5) peonidin 3-glucoside.

6.5 CONCLUSION

This study provides evidence that UAE is an effective technique for the extraction of ACNs from haskap berries using low temperatures and within a reasonable time. Plackett–Burman design showed that the proportion of solvent to dried haskap berries, ethanol concentration, and time of extraction were the most statistically significant variables that affected the UAE of ACNs, quantified by the pH-differential method. In addition, we determined a highly positive and statistically significant relationship between TAC and TPC results. The adequacy of the model obtained by RSM was demonstrated by the close agreement of the predicted response (22.45 mg C3G/g DW) with the experimental result (22.73 mg C3G/g DW) under optimum extraction conditions. It is worth mentioning that one should be careful when using the conditions determined in this study because modifications in variables not included in this study, such as the reaction volume and ultrasound bath power and frequency, could interfere with the results and variations in the optimal parameters may be expected.

Chapter 7 OPTIMIZED ENCAPSULATION OF ANTHOCYANIN-RICH EXTRACT FROM HASKAP BERRIES (*LONICERA CAERULEA* L.) IN CALCIUM-ALGINATE MICROPARTICLES

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Celli, G.B., Ghanem, A., & Brooks, M.S. (2015). Optimized encapsulation of anthocyanin-rich extract from haskap berries (*Lonicera caerulea* L.) in calcium-alginate microparticles. *Journal of Berry Research*, *6*, 1–11.

7.1 ABSTRACT

Background: The chemical instability of extracted anthocyanins (ACNs) limits their application and broader use as food colorants and health-promoting functional ingredients. Encapsulation technology can improve ACN stability and widen their potential applications.

Objective: The objective of this study was to optimize the microencapsulation of ACNs from haskap berries (*Lonicera caerulea* L.) in calcium-alginate particles by the extrusion/gelation method.

Methods: Response Surface Methodology (RSM) by Box–Behnken (BB) design was used for the optimization, followed by the desirability function. Three input variables were evaluated: concentrations of sodium alginate (x_1, w/w %) and calcium chloride (x_2, w/v %), and gelation time (x_3, min). The responses were encapsulation efficiency (y_1, %) and particle size (y_2, μm).

Results: There was a good fit for the model where encapsulation efficiency was used as a separate response (R^2 = 97.98 %), however, the model for particle size did not give as good an agreement (R^2 = 63.86 %). The desirability function was used to optimize the two responses simultaneously and the optimum conditions were determined as 9.0 % (w/w) alginate solution, 2.0 % (w/v) $CaCl_2$, and 10 min in the gelation solution.

Conclusions: These results illustrate the application of RSM followed by a desirability function to optimize encapsulation parameters for a combined response, where several measures are considered.

7.2 INTRODUCTION

Anthocyanins (ACNs) are members of the flavonoid class of secondary metabolites and important water-soluble pigments in higher plants, conferring red, violet, and blue colors to flowers and fruits (Castañeda-Ovando et al., 2009). In addition to their colorant properties, several studies have related the consumption of these compounds to health benefits (Cvorovic et al., 2010; Jennings et al., 2014; Roy et al., 2002), including the reduction of cancer (Gordillo et al., 2009), improved hyperglycemia and insulin resistance (Stull et al., 2010; Vuong et al., 2009), reduction of bone loss in ovariectomized rat model (Devareddy et al., 2008), and neurocognitive benefits (Krikorian et al., 2010). In this context, haskap berries (*Lonicera caerulea* L.) have gained the attention of the scientific community due to their high levels of ACNs and potential antioxidant activity (Celli et al., 2014; Khattab et al., 2015b). These fruits are native to northeastern Asia, where they are recognized as the *elixir of life* by Japanese aboriginals, and were recently introduced in the North American market (Bors et al., 2012). Different health-related benefits have been associated with these berries using *in vitro* and *in vivo* studies (Celli et al., 2014), providing scientific basis for the development of value-added products.

Despite their functionality, the incorporation of ACNs in different food matrices is limited by their stability once extracted due to their electron deficiency (Giusti & Wrolstad, 2003), which is often affected by environmental conditions, such as pH (Hurtado, Morales, González-Miret, Escudero-Gilete, & Heredia, 2009), temperature (Hellström, Mattila, & Karjalainen, 2013), oxygen (Rein, 2005), light (Matsufuji et al., 2007), heat (Wrolstad, 2004), water activity (Garzón & Wrolstad, 2001), and presence of other compounds (e.g. phenolic compounds, enzymes, metal ions, sugars, and ascorbic acid) (Skrede et al., 1992). In this respect, encapsulation is a versatile technology that can address these needs, contributing to the development of high-value food products (Betoret et al., 2011; Celli et al., 2015b).

Encapsulation is a general term to describe any process used to entrap one substance (termed core material or active agent) within another (coating, shell or carrier/wall material) (Zuidam & Shimoni, 2010). Encapsulation techniques can extend a

product's shelf-life (Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008), protecting the active components against degradation during storage (Rascón et al., 2011) and maintaining their functionality (Karathanos, Mourtzinos, Yannakopoulou, & Andrikopoulos, 2007). They can also help to mask unwanted flavors (Martin & Appel, 2010) and increase the effectiveness of natural functional compounds that normally have a lower potency at equivalent levels in comparison to synthetic ingredients (Pegg & Shahidi, 1999). For instance, there is an increasing demand for natural antioxidants that could substitute the synthetic butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary-butyl hydroquinone (TBHQ). However, some natural candidates are not as efficient as the synthetic products when added at the same levels (Almeida-Doria & Regitano-D'Arce, 2000).

The aim of this work was to optimize the encapsulation of an ACN-rich haskap berry extract in calcium-alginate microparticles by the extrusion/gelation method (King, 1988). Firstly, the most appropriate procedure for the preparation of the ACN extract was determined by comparing extraction under stirring conditions with maceration. Then, statistical optimization of the extrusion/gelation process parameters (i.e. sodium alginate and calcium chloride concentrations and gelation time) using individual responses (encapsulation efficiency and particle size) was conducted by Response Surface Methodology (RSM) using Box–Behnken design. This was followed by applying a desirability function to obtain the optimal conditions for the two responses simultaneously.

7.3 MATERIAL AND METHODS

7.3.1 Plant Material

Haskap berries (variety *Indigo Gem*) were used in this study. Berries were mechanically harvested at the Northern Light Orchards (Saskatchewan, Canada) and shipped frozen to LaHave Natural Farms, in Nova Scotia. The berries were then transferred to Dalhousie University and kept at –35 ºC prior to freeze drying. Frozen berries were cut in halves and freeze-dried for approximately three days in a Labconco

FreeZone 2.5 L Bench-top Freeze Dry System (Labconco, Kansas City, MO, USA) (collector temperature was set at –85 ºC and pressure was below 0.100 mbar) (to a low moisture content < 5 %). The freeze-dried samples were stored in a desiccator at –18 ºC until use.

7.3.2 Chemicals

Reagent ethanol, formic and acetic acids, and calcium chloride were purchased from Fisher Scientific (Ottawa, ON, Canada). Alginate Protanal LFR5/60 was kindly donated by FMC BioPolymer (Philadelphia, PA, USA).

7.3.3 Assessment of the Extraction Procedure

The total ACN content obtained from extraction under stirring conditions was compared with maceration using modified methods described by Musa, Abdullah, Jusoh, and Subramaniam (2011), to determine the most appropriate method for preparing the extract. Initial results indicated that there was no significant difference in the extraction yield using freeze-dried berries cut in halves or ground dried berries (particle size < 0.5 mm), possibly due to the delicate structure of these berries (Bors et al., 2012), thus for convenience, freeze-dried berries were used in the extraction without further processing. For extraction under stirring conditions, the freeze-dried samples were combined with 80 % ethanol acidified with 0.5 % formic acid (using a ratio of 1:25, w/v solid:liquid) and stirred at 600 rpm for 10 min at room temperature using a magnetic-stirrer. For maceration, 80 % ethanol acidified with 0.5 % formic acid was added to the freeze-dried berries (same ratio as under stirring conditions) and left for 2 h at room temperature, in the absence of light. In both cases, the mixture was filtered through a Whatman filter paper nº 1 after extraction and kept in a conventional freezer (at –18 ºC) in the absence of light until quantification of the total ACN content. The means of the methods were compared by the unpaired *t*-test using SPSS® version 22 software (IBM, Chicago, IL, USA).

7.3.4 Microencapsulation of Anthocyanin-Rich Extract

Prior to encapsulation, the organic solvent in the extract was removed by a rotary evaporator model BM 400 (Yamato Scientific America Inc., Santa Clara, CA, USA) with a water bath kept at $T < 50$ ºC, equipped with a vacuum pump V-700 (Büchi Labortechnik AG, Flawil, Switerzland) and a water circulation cooler WK 230 (Lauda, Lauda-Königshofen, Germany). The resultant aqueous fraction was kept at –18 ºC protected from light until further use.

A system consisting of a mini-pump with variable flow rate (Fisher Scientific) and a 26 G needle was used for the encapsulation of ACN-rich extract in calcium-alginate microparticles by the extrusion/gelation method (Figure 7.1) adapted from Santos, Albarelli, Beppu, and Meireles (2013). The pH of the aqueous fraction was low (approximately 2.9) and its adjustment was essential to prevent immediate gelation of the alginate solution and precipitation of alginic acid. The pH of the extract was thus adjusted to approximately 4.5 with 0.1 N NaOH prior to mixing with the alginate solution. The ratio extract:alginate solution was set at 1:4 (v/v) based on preliminary results, taking into account the concentration of ACNs in the evaporated extract. The extract/alginate solution was then extruded through the needle into 30 mL of $CaCl_2$ gelation medium, using an average flow rate of 0.3 mL/min. The distance between the tip of the needle and the gelation medium surface was fixed at 10 cm. The alginate membrane formed immediately upon contact of the solution with the $CaCl_2$ medium. After the gelation time, particles were recovered by vacuum filtration, left for 1 min in 10 mL of distilled water, and then dried overnight in the dark at room temperature.

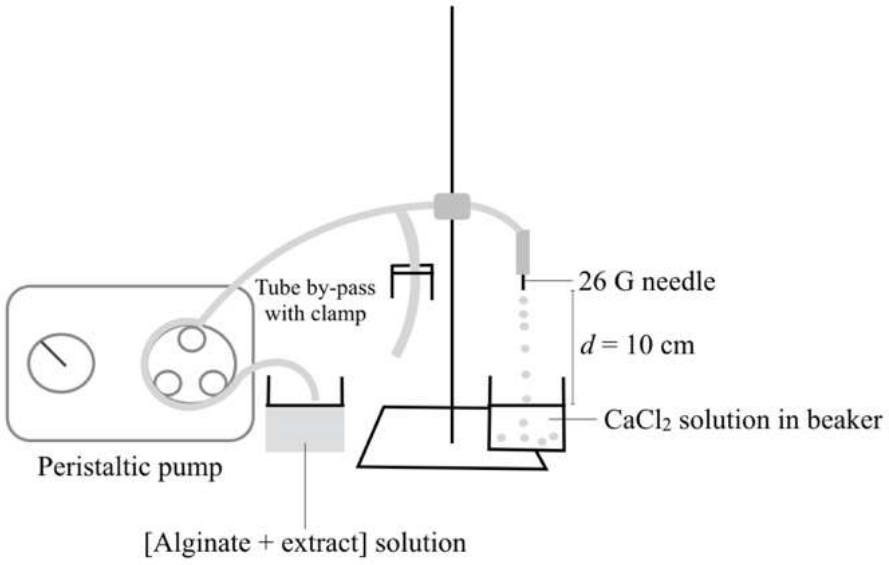

Figure 7.1 Diagram of the system used for the preparation of alginate microparticles. A tube by-pass with clamp was used for further adjustment of the flow-rate

7.3.5 Experimental Design and Data Analysis

7.3.5.1 Box–Behnken Design for Optimization of Encapsulation

A Box–Behnken design with three levels (–1, 0, +1, representing low, middle, and high levels) was used to optimize the encapsulation of ACN-rich extract from haskap berries. The following variables and levels were considered in this study, based on previous results and reports from the literature (Ma et al., 2008; Santos et al., 2013): sodium alginate concentration (ξ_1), calcium chloride concentration acidified with 2 % (v/v) acetic acid glacial (ξ_2,), and time in the gelation solution (ξ_3) (Table 7.1).

Table 7.1 Input variables (natural and coded) and levels chosen for optimization

Input variable	Coded variable	Levels		
		–1	0	+1
Sodium alginate concentration (% w/w) (ξ_1)	$x_1 = (\xi_1 - 9.5)/0.5$	9	9.5	10
$CaCl_2$ concentration (% w/v) (ξ_2)	$x_2 = (\xi_2 - 2.5)/0.5$	2	2.5	3
Gelation time (min) (ξ_3)	$x_3 = (\xi_3 - 20)/10$	10	20	30

The optimization consisted of 15 runs (with a triplicate in the center point) and the design matrix is presented in Table 7.2. The responses assessed were encapsulation efficiency (y_1, %) and size of the dried particles (y_2, μm). Design-Expert® version 9.0 software (Stat-Ease, Inc., Minneapolis, MN, USA) was used for analysis and modeling of the responses. A second-degree polynomial equation was fitted for each response variable, generically expressed as:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_i \sum_{j=i+1} \beta_{ij} x_i x_j \quad (32)$$

where y is the response; β_0 is the constant (or model intercept); β_i is the linear coefficient of the coded variables; β_{ii} is the quadratic coefficient; β_{ij} the cross-product coefficient; x_i and x_j are the coded independent variables.

7.3.5.2 Desirability Function

The desirability function approach (Derringer & Suich, 1980) was used to optimize the responses simultaneously and to identify the ideal conditions for the preparation of ACN encapsulates. This function involved two phases: (a) each response was transformed into a desirable value (d_i), which can assume values from 0 to 1; and (b) based on the individual desirability values, the overall desirability (D) for all dependent variables was determined.

Table 7.2 Uncoded Box–Behnken design matrix with experimental results

No.[a]	ξ_1	ξ_2	ξ_3	y_1	y_2
1	9.0	2.0	20	42.25	1,119.5
2	10.0	2.0	20	46.20	1,125.1
3	9.0	3.0	20	17.97	1,119.6
4	10.0	3.0	20	50.66	1,129.3
5	9.0	2.5	10	57.84	1,113.5
6	10.0	2.5	10	63.12	1,123.6
7	9.0	2.5	30	39.39	1,120.2
8	10.0	2.5	30	62.27	1,121.9
9	9.5	2.0	10	51.85	1,118.5
10	9.5	3.0	10	46.50	1,124.3
11	9.5	2.0	30	36.79	1,115.8
12	9.5	3.0	30	32.32	1,114.6
13	9.5	2.5	20	30.57	1,121.3
14	9.5	2.5	20	27.90	1,126.1
15	9.5	2.5	20	29.33	1,125.2

Note: [a]Number was used for identification purposes only and does not indicate the order in which the experimental runs were conducted; ξ_1 – sodium alginate concentration (%); ξ_2 – $CaCl_2$ concentration (%); ξ_3 – gelation time (min); y_1 – encapsulation efficiency (%); y_2 – size (μm)

The following scenarios were considered when maximizing encapsulation efficiency and minimizing size (Derringer & Suich, 1980):

Maximization (for encapsulation efficiency):

$$d_i = \begin{cases} 0 & y_i \le y_{i,min} \\ \left[\frac{y_i - y_{i,min}}{y_{i,max} - y_{i,min}}\right]^r & y_{i,min} < y_i < y_{i,max} \\ 1 & y_i \ge y_{i,max} \end{cases} \qquad \text{for } i = 1,2,\ldots,k$$

Consequently,

$d_i = 0$ response is smaller than the minimum value

$0 \leq d_i \leq 1$ response is between minimum and maximum values

$d_i = 1$ response is greater than the maximum value

Minimization (for size):

$$d_i = \begin{cases} 1 & y_i \leq y_{i,min} \\ \left[\frac{y_i - y_{i,max}}{y_{i,min} - y_{i,max}}\right]^r & y_{i,min} < y_i < y_{i,max} \\ 0 & y_i \geq y_{i,max} \end{cases} \quad \text{for } i = 1,2,\ldots,k$$

Consequently,

$d_i = 0$ response is greater than the maximum value

$0 \leq d_i \leq 1$ response is between minimum and maximum values

$d_i = 1$ response is smaller than the minimum value

where $y_{i,min}$ and $y_{i,max}$ are the minimum and maximum acceptable values of the response (y_i), respectively, and r is the weight factor that determines the shape of the desirability graph. The r and importance (w) values were set at 1 and 3, respectively, where the importance refers to the effect of each response on the overall desirability.

Once the d_i values were found, the calculation of their geometric mean resulted in the overall desirability (D), as follows:

$$D = \left(d_1^{w_1} x d_2^{w_2} x \ldots d_k^{w_k}\right)^{1/\sum_i^k w_i} \quad (33)$$

where k is the number of variables being optimized.

7.3.5.3 Responses Assessed

Encapsulation efficiency or *EE* (y_1, %) was indirectly assessed by measuring the total anthocyanin content in the recovered $CaCl_2$ solution (i.e. amount not entrapped) by the pH-differential method (Giusti & Wrolstad, 2001). Absorbance values were measured at λ_{max} and 700 nm against distilled water using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Madison, WI, USA). These analyses were performed in triplicate. As the particles were not completely spherical, the size of the dry particles (y_2, μm) was determined by measuring the major axis of individual particles using a caliper ruler and reporting the average measurement from 10 particles.

7.4 RESULTS AND DISCUSSION

Two methods for extraction were evaluated in this study. Extraction under stirring and by maceration at room temperature yielded 20.74 ± 1.07 and 18.09 ± 1.19 mg cyanidin 3-glucoside equivalents/g of dried berries by the pH-differential method. No significant difference was observed between the methods in the unpaired *t*-test ($p > 0.05$). Reports in the literature have indicated significant differences between similar extraction methods. For example, Musa et al. (2011) found that stirring guava fruit with solvent for 1 h at 1000 rpm resulted in significantly higher antioxidant activity than maceration for 1 and 2 days. Adjé et al. (2010) also showed that maceration under stirring resulted in higher recovery of polyphenols and ACNs from *Delonix regia* tree flower than maceration alone. However, this is very much determined by the range of extraction parameters used, and it was not the aim of this study to optimize the extraction procedure. For this reason, the stirring method was selected for the preparation of the extract for encapsulation as similar ACN recovery was obtained in a shorter period of time.

Alginate particles are commonly formed by extruding a solution of sodium or potassium alginate through a needle or nozzle into a gelation solution containing calcium ions (Kuo & Ma, 2001). The alginate chains are crosslinked by the divalent cations in the so-called egg box model and the reader is referred to Juárez, Spasojevic, Faas, and de Vos (2014) and Pistone, Qoragllu, Smistad, and Hiorth (2015) for a schematic

representation of this model. This is a mild encapsulation method that could contribute to the retention and delivery of ACNs in food products. According to the manufacturer, the sodium alginate used in this study contained approximately 65–75 % of guluronic acid groups. Martinsen, Skjåk-Bræk, and Smidsrød (1989) showed that particles with highest mechanical strength, lowest shrinkage, and highest porosity were obtained with alginate containing more than 70 % of guluronic acid. An example of the particles produced is presented in Figure 7.2.

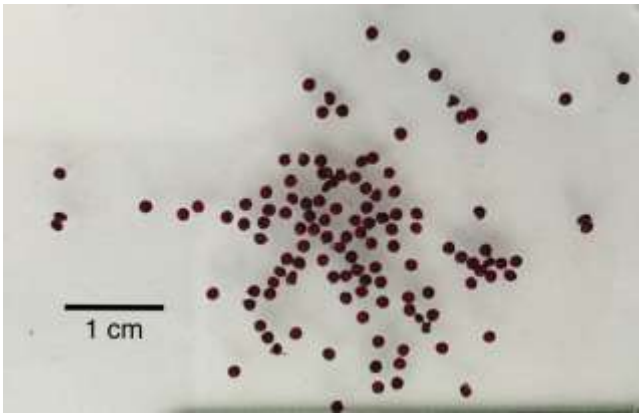

Figure 7.2 An example of the particles produced during the optimization after being left to dry overnight at room temperature in the absence of light

The ANOVA results showing the significant variables ($p < 0.05$) for the encapsulation efficiency of ACNs and size of calcium-alginate particles analyzed by RSM are presented in Tables 7.3 and 7.4, respectively. The results indicated that the models were significant for the responses analyzed ($F_{EE} = 48.39$ and $F_{Size} = 6.48$, $p < 0.05$). The non-significant lack-of-fit ($p > 0.05$) for both models also indicated that they fitted the experimental data. The models explained 97.98 and 63.86 % of the variations in *EE* and particle size, respectively. The model for size was not as good as the one for *EE* (low R^2 and large discrepancies between adjusted and predicted R^2); however, the coefficients of variation (CV% = 6.37 and 2.51 for *EE* and size, respectively) indicated an acceptable level of precision and reliability of the models.

Table 7.3 ANOVA results for the model with encapsulation efficiency (y_1) as the response

Source	***df***	**SS**	**MS**	***F*-value**	***p*-value**	***P* (%)**
Model	7	2460.46	351.49	48.39	<0.0001	97.98
x_1	1	525.07	525.07	72.29	<0.0001	20.91
x_2	1	109.78	109.78	15.11	0.0060	4.37
x_3	1	294.37	294.37	40.53	0.0004	11.72
x_1x_2	1	206.42	206.42	28.42	0.0011	8.22
x_1x_3	1	77.42	77.42	10.66	0.0138	3.08
x_1^2	1	538.72	538.72	74.17	<0.0001	21.45
x_3^2	1	795.86	795.86	109.57	<0.0001	31.69
Error	7	50.84	7.26			2.02
Lack-of-fit	5	47.27	9.45	5.29	0.1665	1.88
Pure error	2	3.57	1.79			0.14
Total	14	2511.30				100.00
S	2.70		Adjusted R^2	0.9595		
R^2	0.9798		Predicted R^2	0.8590		

Note: x_1 – alginate concentration; x_2 – $CaCl_2$ concentration; x_3 – time in the gelation solution; *P* – percentage contributions; *S* – standard error of the regression; R^2 – regression coefficient

In relation to the terms in the model, x_3 had to be included in the model for particle size for hierarchical purposes (Table 7.4). The percentage contribution (*P*) was calculated for all terms by dividing the sum of squares (*SS*) of each component by the total *SS* and these results indicated that x_1, x_1^2, and x_3^2 affected the encapsulation efficiency by 20.91, 21.45, and 31.6 %, respectively. In the case of size, the variables x_1 and x_3^2 affected the particle size by 32.50 and 28.93 %, respectively.

Table 7.4 ANOVA results for the model with particle size (y_2) as the response

Source	*df*	SS	MS	*F*-value	*p*-value	*P* (%)
Model	3	180.37	60.12	6.48	0.0087	63.86
x_1	1	91.80	91.80	9.89	0.0093	32.50
x_3	1	6.84	6.84	0.74	0.4087	2.42
x_3^2	1	81.72	81.72	8.81	0.0128	28.93
Error	11	102.07	9.28			36.14
Lack-of-fit	9	89.05	9.89	1.52	0.4589	31.53
Pure error	2	13.02	6.51			4.61
Total	14	282.43				100.00
S	3.05		Adjusted R^2	0.5401		
R^2	0.6386		Predicted R^2	0.2650		

Note: x_1 – alginate concentration; x_2 – $CaCl_2$ concentration; x_3 – time in the gelation solution; *P* – percentage contributions; *S* – standard error of the regression; R^2 – regression coefficient

The quadratic models for each response variable are described in coded and natural variables, as follows:

$$y_1 = 28.10 + 8.10x_1 - 3.70x_2 - 6.07x_3 + 7.18x_1x_2 + 4.40x_1x_3 + 12.04x_1^2 + 14.64x_3^2 \quad (34)$$

$$EE\ (\%) = 5160.62 - 988.52\xi_1 - 280.39\xi_2 - 14.82\xi_3 + 28.73\xi_1\xi_2 + 0.88\xi_1\xi_3 + 48.17\xi_1^2 + 0.15\xi_3^2 \quad (35)$$

$$y_2 = 1123.73 + 3.39x_1 - 0.92x_3 - 4.68x_3^2 \quad (36)$$

$$Size\ (\mu m) = 1042.50 + 6.77\xi_1 + 1.78\xi_3 - 0.05\xi_3^2 \quad (37)$$

The surface graphs for the responses evaluated in relation to the variables presented in the model are illustrated in Figure 7.3. The encapsulation efficiency ranged from 17.97 to 63.12 %. Figure 7.3A shows that higher encapsulation efficiency (63.12 %) could be obtained with a high concentration of alginate (10 %, w/w) and shorter period of

time (10 min) in the crosslinking solution, keeping the concentration of $CaCl_2$ in its center point (2.5 %, w/v). When the concentration of alginate was decreased to 9 % (w/w) (keeping the other variables constant), the *EE* dropped considerably (17.97 %), providing evidence that this variable played an important role in *EE*. This was also demonstrated by Lotfipour, Mirzaeei, and Maghsoodi (2012), who reported a significant effect of alginate concentration on the *EE* of probiotics using the extrusion method, whereas the concentration of $CaCl_2$ and gelation time were considered insignificant. A higher concentration of alginate could have blocked the diffusion of encapsulated ACNs due to a denser and thicker membrane with greater availability of active sites for crosslinking with calcium (Mandal, Kumar, Krishnamoorthy, & Basu, 2010); however, this would be dependent on the concentration of calcium ions and their availability to crosslink the alginate chains and also the time the particles remained in the gelation medium. ACNs are hydrophilic compounds and it is possible that they could have diffused out from the calcium-alginate particles if they remained for too long in the $CaCl_2$ solution.

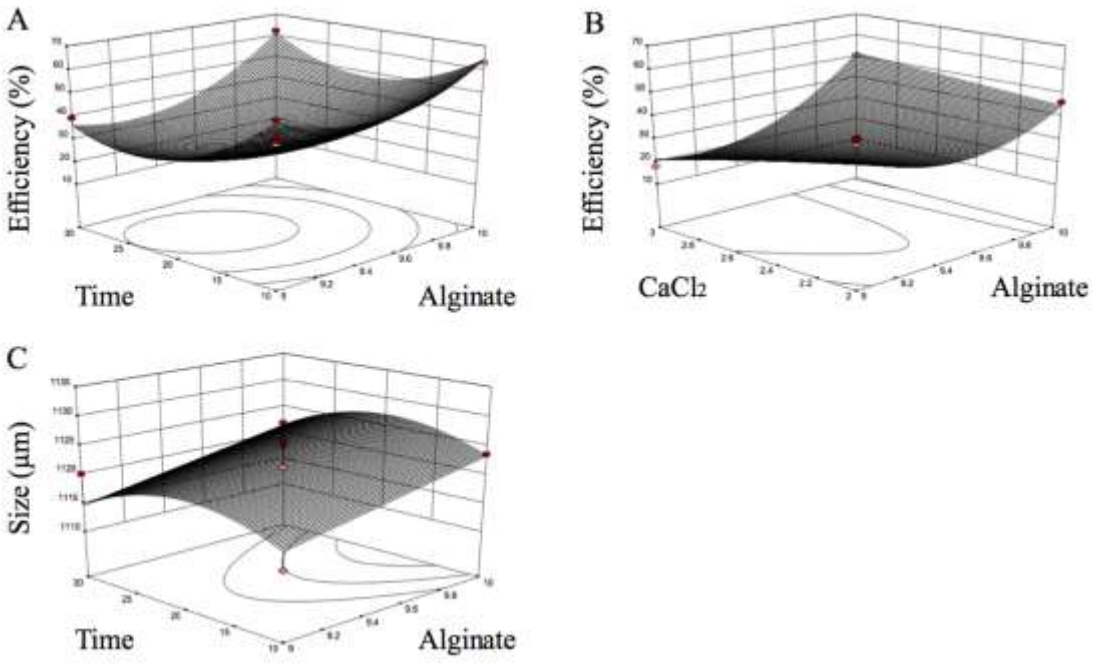

Figure 7.3 Surface plots of the two responses assessed (A and B – encapsulation efficiency; C – size) versus the variables included in the model

At a low concentration of alginate (9 %, w/w), an increase of $CaCl_2$ concentration from 2 to 3 % (w/v) resulted in a reduction of *EE* from 42.25 to 17.97 %. The gelation was carried out under an acidic gelling environment to prevent the degradation of ACNs, and the flavylium cation was most likely the ACN form found during encapsulation (Brouillard & Dubois, 1977; Castañeda-Ovando et al., 2009). It could have been possible that higher concentrations of $CaCl_2$ could have led to an electrostatic repulsion and attraction by calcium and chlorine ions, respectively, which could have also contributed to the leakage of ACNs from the particles. Jiang et al. (2007) also showed that higher concentrations of $CaCl_2$ resulted in lower encapsulation efficiency of β-glucuronidase in alginate particles.

In relation to size, larger particles were obtained with higher concentrations of alginate (10 %, w/w) around the center point for time (20 min). The concentration of alginate is intimately related to the viscosity of the mixture (Klokk & Melvik, 2002; Liu et al., 2003) and the behavior during extrusion. Even though viscosity was not evaluated in this study, a subjective assessment of the solutions indicated a positive correlation between polymer concentration and viscosity. A more viscous solution would reduce the flux and increase the detachment time of droplets from the needle using the same flow rate and pressure, resulting in larger particles. Lotfipour et al. (2012) also showed that the concentration of alginate had a significant effect on the particles produced by extrusion. It is likely that the time spent by the particles in the gelling solution would impact the formation of the internal network structure within the alginate gel and, consequently, affect the mechanical resistance of the particles, although it was not assessed in this work. Deladino, Anbinder, Navarro, and Martino (2008) showed that dried calcium-alginate particles prepared with yerba mate extract had a spherical disc shape with a collapsed center, resembling a red blood cell. In this study, the particles also had a disc shape, but no collapse was observed. This collapse during drying has been associated with the heterogeneous gelation mechanism where a dense surface layer and loose core would be observed (Deladino et al., 2008; Shu & Zhu, 2002), which was not the case in the present study.

In order to optimize the two responses simultaneously, the desirability function methodology was used in which *EE* was maximized, whereas size was minimized. The

individual desirability values for *EE* and size were approximately 1.00 and 0.81, respectively. The overall desirability (*D*) was approximately 0.90, with predicted *EE* of 68.03 % and size of 1,116.6 μm. Using this strategy, the optimization of the two responses was predicted at the following conditions: alginate concentration of 9.0 % (w/w), $CaCl_2$ concentration of 2.0 % (w/v), and 10 min in the gelation solution.

7.5 CONCLUSION

The encapsulation of ACN-rich extract from haskap berries (*Lonicera caerulea* L.) in calcium-alginate particles was optimized by RSM using Box–Behnken design. Three input variables (alginate and $CaCl_2$ concentrations, and time for crosslinking) were investigated and their impacts on *EE* and size were assessed. Using the desirability function, it was possible to optimize the two responses simultaneously. The optimum conditions were determined as 9.0 % (w/w) alginate solution, 2.0 % (w/v) $CaCl_2$, and 10 min in the gelation solution. Further investigation is required to determine the release profile of the ACNs from the particles and the stability of the encapsulated ACNs under adverse conditions.

Chapter 8 DEVELOPMENT AND EVALUATION OF FLOATING ALGINATE MICROSPHERES FOR ORAL DELIVERY OF ANTHOCYANINS

Materials of this chapter have been submitted for publication in **Journal of Microencapsulation**.

Celli, G.B., Ghanem, A., & Brooks, M.S. (2016). Development and evaluation of floating alginate microparticles for the oral delivery of anthocyanins. In Review: *Journal of Microencapsulation*.

8.1 ABSTRACT

The goal of this study was to develop floating microspheres that could be used as gastroretentive systems for the delivery of anthocyanins (ACNs). These compounds are absorbed in the stomach and initial section of the small intestine, and insufficient residence time in these organs could result in limited absorption and contribute to degradation. The microparticles were prepared by ionotropic gelation of alginate (9 %, w/w) with calcium ions ($CaCl_2$ at 2 %, w/v), with calcium carbonate as the gas-generating compound. The effect of acetic acid concentration (2 and 10 %, v/v) in the gelation medium was investigated. Increasing the carbonate:alginate weight ratio from 0 to 3:4 resulted in different degrees of floatability, larger particles, higher encapsulation efficiency, and lower ACN release. The power law equation fitted the experimental data well, indicating that release occurred mainly by diffusion. This is the first time floating microspheres are proposed as gastroretentive platforms for the delivery of ACNs.

8.2 INTRODUCTION

Gastroretentive systems (GRS) differ from oral conventional delivery platforms as the time they remain in the stomach is longer (Joseph et al., 2002). For instance, Ma et al. (2008) showed that floating particles remained in the stomach of volunteers for over 5 h compared to a residence time of 2.5 h for non-floating systems. These systems can increase the residence time of bioactive compounds that are stable under acidic

conditions and absorbed in the stomach, such as anthocyanins (ACNs), a group of hydrophilic pigments (Delgado-Vargas et al., 2000) with health-promoting properties (Cassidy et al., 2013; Wallace, 2011). Amongst the known sources of these compounds, haskap berries (*Lonicera caerulea* L.) exhibit a high concentration of ACNs in addition to other bioactive molecules (Celli et al., 2014), which makes them an interesting raw material for the development of value-added products.

Although the stomach is not often considered an absorption site, researchers have shown that ACNs are absorbed in this organ and in the initial portion of the small intestine (Passamonti et al., 2003; Celli et al., 2016), which could explain the rapid appearance of these compounds in the circulation (within 30 min after administration) (Vanzo et al., 2011). In general, ACNs are stable in the stomach (pH < 2) (Liang et al., 2012) and likely found in the stable flavylium cation form (Brouillard & Dubois, 1977). This ion has limited chances of being absorbed by passive diffusion (Lipinski et al., 1997), and bilitranslocase found in the gastric mucosa has been suggested as the carrier responsible for its absorption (Passamonti et al., 2003). This protein is primarily involved in the hepatic detoxification process and transport of organic anions from the circulation into the hepatocytes (Battiston et al., 1999). However, under certain conditions, bilitranslocase can reach saturation, i.e. an increase in the amount of ACNs administered does not result in a proportionally higher bioavailability, as demonstrated by Adisakwattana et al. (2011) and Keane et al. (2015). The ACNs that are not absorbed in the upper gastrointestinal (GI) tract are subject to extensive metabolism (Czank et al., 2013) and degradation (Liu et al., 2014).

The objective of the present study was to develop floating microparticles for the oral delivery of ACNs extracted from haskap berries, using sodium alginate, calcium chloride, and calcium carbonate ($CaCO_3$) as the hydrocolloid, crosslinking ion, and gas-generating compound, respectively. $CaCO_3$ releases carbon dioxide gas when reacted with acid, which permeates the alginate gel matrix forming bubbles or pores (Choi, Park, Hwang, & Park, 2002), allowing the particles to float. Different $CaCO_3$:alginate weight ratios were investigated and their effects on the encapsulate properties (yield, particle size, encapsulation efficiency, and floating capacity) and release profiles were assessed in comparison to the control (non-floating particles without $CaCO_3$).

8.3 MATERIAL AND METHODS

8.3.1 Materials

Formic, acetic, and hydrochloric acids, reagent ethanol, calcium carbonate, potassium chloride, sodium acetate anhydrous, Tween® 80, and sodium citrate were purchased from Fisher Scientific (Ottawa, ON, Canada). Calcium chloride 96 % anhydrous and calcium carbonate 98 % pure were purchased from Acros Organics (Fairlawn, NJ, USA). Low-viscosity sodium alginate Protanal LFR5/60 (65-75 % guluronic acid) was kindly donated by FMC BioPolymer (Philadelphia, PA, USA).

8.3.2 Plant Material

Frozen haskap berries (var. *Indigo Gem*) were used in this study. The berries were halved and freeze-dried in a Labconco FreeZone 2.5 L Bench-top Freeze Dry System (Labconco, Kansas City, MO, USA) (final moisture content < 5 %, w/w). The freeze-dried berries were stored in a desiccator at –18 ºC until extraction.

8.3.3 Preparation of Anthocyanin-Rich Freeze-Dried Haskap Extract

The extraction of ACNs from freeze-dried haskap berries was conducted according to Celli, Ghanem, and Brooks (2015c). Freeze-dried samples were extracted with 80 % ethanol acidified with 0.5 % formic acid in a solid to solvent ratio of 1:25 (w/v), under magnetic stirring at 600 rpm for 10 min at room temperature. The mixture was filtered through a Whatman filter paper no. 1 and the extract was kept at –18 ºC in the absence of light until further use.

The organic solvent was removed prior to the fractionation under vacuum (rotary evaporator model RE-51, Yamato Scientific America Inc., Santa Clara, CA, USA) kept at $T < 50$ ºC, equipped with a vacuum pump V-700 (Büchi Labortechnik AG, Flawal, Switzerland) and a water circulation cooler WKL 230 (Lauda, Lauda-Königshofen, Germany). The resultant fraction was kept at –18 ºC until further use. The fractionation of

the evaporated extract was performed according to Kalt et al. (2008b) with some modifications. Briefly, the evaporated extract was added to a C18 column (previously washed with water) and washed with approximately two volumes of water to remove sugars, followed by the elution of bound components with 1.5 volume of reagent ethanol (acidified with 0.5 % formic acid). The solvent of the ACN-rich fraction was removed by rotary evaporation and freeze drying at the same conditions as before (resulting in the purification of ACNs in the resultant powder by 22-fold), and the resultant powder was stored at –18 ºC prior to use.

8.3.4 Preparation of Calcium-Alginate Microparticles

Microparticles with or without $CaCO_3$ were prepared according to the procedure by Celli et al. (2015c) with some modifications. Freeze-dried extract was dissolved in distilled water at a concentration of 2 mg/mL under magnetic stirring. The pH was adjusted to approximately 4.8 with sodium citrate to prevent the gelation of alginate and precipitation as alginic acid. Then, sodium alginate was dissolved in this solution (9.0 %, w/w) and stirred thoroughly for at least 2 h. For the preparation of floating particles, $CaCO_3$ was added to the alginate solution at different weight ratios ($CaCO_3$:alginate = 1:4, 1:2, and 3:4, w/w), according to Choi et al. (2002). The gelation medium consisted of $CaCl_2$ (2.0 %, w/v) in 2 % (v/v) or 10 % (v/v) glacial acetic acid. The alginate solution was then extruded through a 26 G needle using a Fisher Syringe Pump Model 78-0100I (Fisher Scientific, Holliston, MA, USA) at an average flow rate of 1.0 mL/min into 30 mL of gelation medium, gently stirred (60 rpm). The distance between the tip of the needle and the gelation medium surface was fixed at 10 cm. The gelation was allowed to proceed for 10 min under stirring, after which the particles were recovered by vacuum filtration and washed with distilled water. Both the formation of the microspheres and the gelation were carried out at room temperature. Particles were dried at room temperature in the absence of light until constant weight was observed.

8.3.5 Particle Characterization

8.3.5.1 Determination of Particle Size

The particle size was determined using a caliper ruler. As the particles were not completely spherical, the size of the fresh and dry particles was determined by measuring the major axis of individual particles and reporting the average measurement from 10 particles. Wet and dry particles were measured to determine the degree of shrinkage after drying.

8.3.5.2 *In Vitro* Floating Capacity

The *in vitro* floating capacity was assessed by the visual observation method described by El-Gibaly (2002), slightly modified. Using a water bath shaker Model 2870 (Thermo Scientific), a fixed amount of individual microspheres (20) was placed into 100 ml of enzyme-free simulated gastric fluid (0.1 N HCl containing 0.02 % w/v Tween® 80) and maintained at 37 ± 0.5 ºC, undisturbed. The floating lag time was determined by measuring the time it took for the encapsulates to rise to the surface and float. Both the percentage of floating particles (counted visually) and the duration of buoyancy (time during which the particles remained buoyant on the simulated gastric solution) (n = 3) were determined at fixed time intervals during an 8-h period.

8.3.5.3 Determination of Anthocyanin Encapsulation Efficiency

Encapsulation efficiency (*EE*, %) was indirectly assessed by measuring the ACN content in the recovered $CaCl_2$ solution (i.e. amount not entrapped) by the pH-differential method (Giusti & Wrolstad, 2001). Absorbances were measured at λ_{max} (determined as 515 nm) and 700 nm in a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Madison, WI, USA) against distilled water (blank).

8.3.6 *In Vitro* Anthocyanin Release

The release of ACNs from the floating particles was determined by the method described by Santos et al. (2013), with modifications. A known weight of particles (100 mg) was added to 150 mL enzyme-free simulated gastric fluid (0.1 N HCl containing 0.02 % w/v Tween® 80) maintained at 37 ± 0.5 ºC in a reciprocal shaker bath Model 2870 (Thermo Scientific), agitated at 95 cycles per minute. At specific time intervals, an aliquot of 5 mL was removed and replaced by the same volume of fresh media (at 37 ± 0.5 ºC). Samples were then filtered through 0.45 µm syringe filter and analyzed spectrophotometrically at λ_{max} and 700 nm against a blank (distilled water) and a known concentration of ACNs in simulated gastric fluid.

ACN release kinetics were analysed by plotting the mean release data (%) *vs.* time (h). Curves were fitted for Peppas (or power law) equation (Ritger & Peppas, 1987; Siepmann & Peppas, 2001) using SigmaPlot™ version 13.0 software (Systat Software, Inc., Sao Jose, CA, USA), as follows:

$$\frac{M_t}{M_\infty} = kt^n \tag{38}$$

where M_t and M_∞ are the cumulative absolute amount of drug released at time t and infinite time, respectively; k is the kinetic constant; and n is the release exponent that characterizes the diffusional mechanism.

The difference in release profiles with the addition of $CaCO_3$ was assessed by the similarity (f_2) and difference (f_1) factors (Moore & Flanner, 1996, referenced by Shah, Tsong, Sathe, & Liu, 1998), calculated using the following equations:

$$f_2 = 50 \cdot log\left\{\left[1 + \left(\frac{1}{P_t}\right)\sum_{i=1}^{P_t}(R_i - T_i)^2\right]^{-1/2} \cdot 100\right\} \tag{39}$$

$$f_1 = \left\{\left[\sum_{i=1}^{P_t}|R_i - T_i|\right]/\sum_{i=1}^{P_t} R_i\right\} \cdot 100 \tag{40}$$

where P_t is the number of time points, and R_i and T_i are the release value of the reference and test at time i, respectively. The release profiles were considered significantly different if $f_2 < 50$. The f_1 increases proportionally with the dissimilarity between the curves.

8.3.7 Statistical Analysis

All the experiments were conducted in triplicate unless otherwise stated and results were reported as mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS® version 22 software (IBM Corp., Armonk, NY, USA). Tukey's test ($p = 0.05$) was used to assess significant differences between means.

8.4 RESULTS

8.4.1 Determination of Particle Size

Different $CaCO_3$:alginate weight ratios were investigated for the preparation of floating particles and comparison with non-floating (control) microspheres. Figure 8.1 shows that increasing the ratio of $CaCO_3$ significantly increased the size of the microspheres in comparison to the controls (with the exception of 1:4 ratio prepared with 2 % acetic acid), in agreement with the results found by Choi et al. (2002). The use of different concentrations of acetic acid also impacted particle size: microspheres extruded into 10 % acetic acid solution were significantly ($p < 0.05$) larger than the ones prepared with 2 % acetic acid.

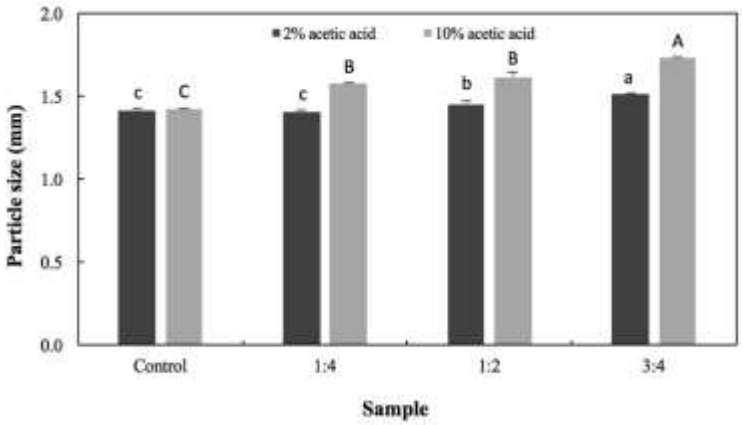

Figure 8.1 Particle size of dry microspheres prepared with different $CaCO_3$:alginate ratios (1:4, 1:2, and 3:4) in comparison to the control (no carbonate added). Bars represent mean ± standard deviation ($n = 3$). Those identified with the same lower- and uppercase letters are not significantly different at $p < 0.05$, according to the Tukey's test, for gelation media containing 2 and 10 % glacial acetic acid, respectively

8.4.2 *In Vitro* Floating Capacity

The floating capacity of the dry microspheres was evaluated in enzyme-free simulated gastric fluid. The control microspheres sank uniformly in the simulated fluid and were statistically different ($p < 0.05$) than the other samples. Particles containing $CaCO_3$ prepared with 10 % acetic acid floated immediately when placed in a simulated gastric fluid, whereas those prepared with a lower concentration of acid floated after variable periods of time (Figure 8.2). The floating capacity persisted over the duration of the experiment (8 h).

8.4.3 Determination of ACN Encapsulation Efficiency

Taking into consideration the colour intensity of the particles produced, the macroscopic appearance of the particles suggested that those prepared with 10 % acetic acid had lower *EE* (i.e. the red colour is less intense than the microspheres prepared with 2 % acetic acid) (Figure 8.3). This preliminary observation was confirmed by spectrophotometric measurement: the *EE* of control particles were 61.42±1.90 % and 57.04±3.66 for samples prepared with 2 and 10 % acetic acid, respectively.

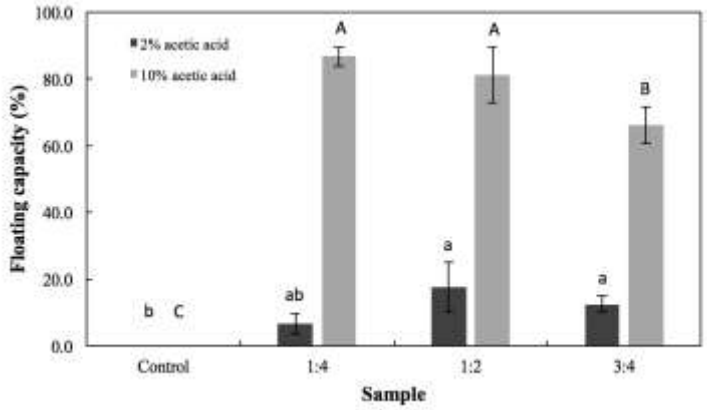

Figure 8.2 Floating capacity of dry microspheres prepared with different $CaCO_3$:alginate ratios (1:4, 1:2, and 3:4) in comparison to the control. Bars represent mean ± standard deviation (n = 3). Those identified with the same lower- and uppercase letters are not significantly different at $p <$ 0.05, according to the Tukey's test, for gelation media containing 2 and 10 % glacial acetic acid, respectively

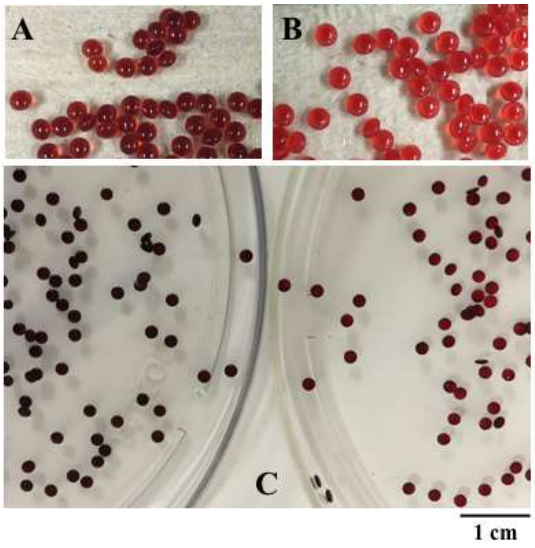

Figure 8.3 Visual observation of fresh non-floating microspheres prepared with (A) 2 % and (B) 10 % acetic acid, and (C) after drying (2 % – left, 10 % – right) at room temperature shows the difference in *EE*, as indicated by the colour of the particles

Unlike previous reports (Shishu, Gupta, & Aggarwal, 2007), the addition of $CaCO_3$ significantly increased the *EE*, specially for the microspheres gelled in cross-linking bath containing 2 % acetic acid (Figure 8.4), which could indicate that the

calcium ions released from the carbonate contributed to the internal gelation of the alginate chains.

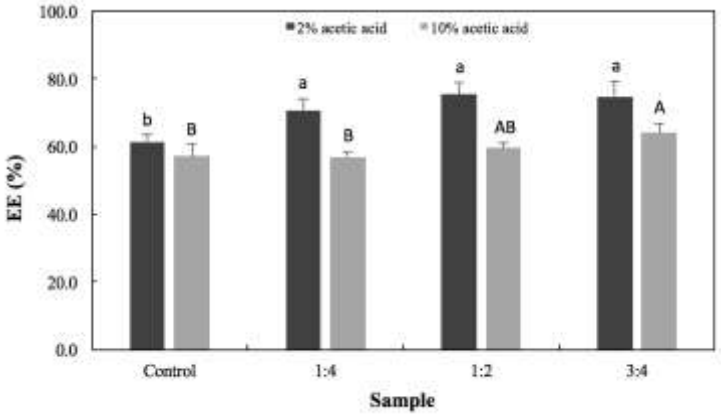

Figure 8.4 *EE* of microspheres prepared with different $CaCO_3$:alginate ratios (1:4, 1:2, and 3:4) in comparison to the control (no carbonate added). Bars represent mean ± standard deviation (n = 3). Those identified with the same lower- and uppercase letters are not significantly different at $p <$ 0.05, according to the Tukey's test, for gelation media containing 2 and 10 % glacial acetic acid, respectively

8.4.4 *In Vitro* Anthocyanin Release

The release profiles of the control and floating microspheres in enzyme-free simulated gastric fluid are shown in Figure 8.5. In general, all the samples exhibited a burst phase (up to 15 min) with high amounts of ACNs released into the acidic fluid, followed by a reduction in release rate. For both gelation media, increased concentrations of $CaCO_3$ resulted in lower concentrations of ACNs released over time until a plateau was reached.

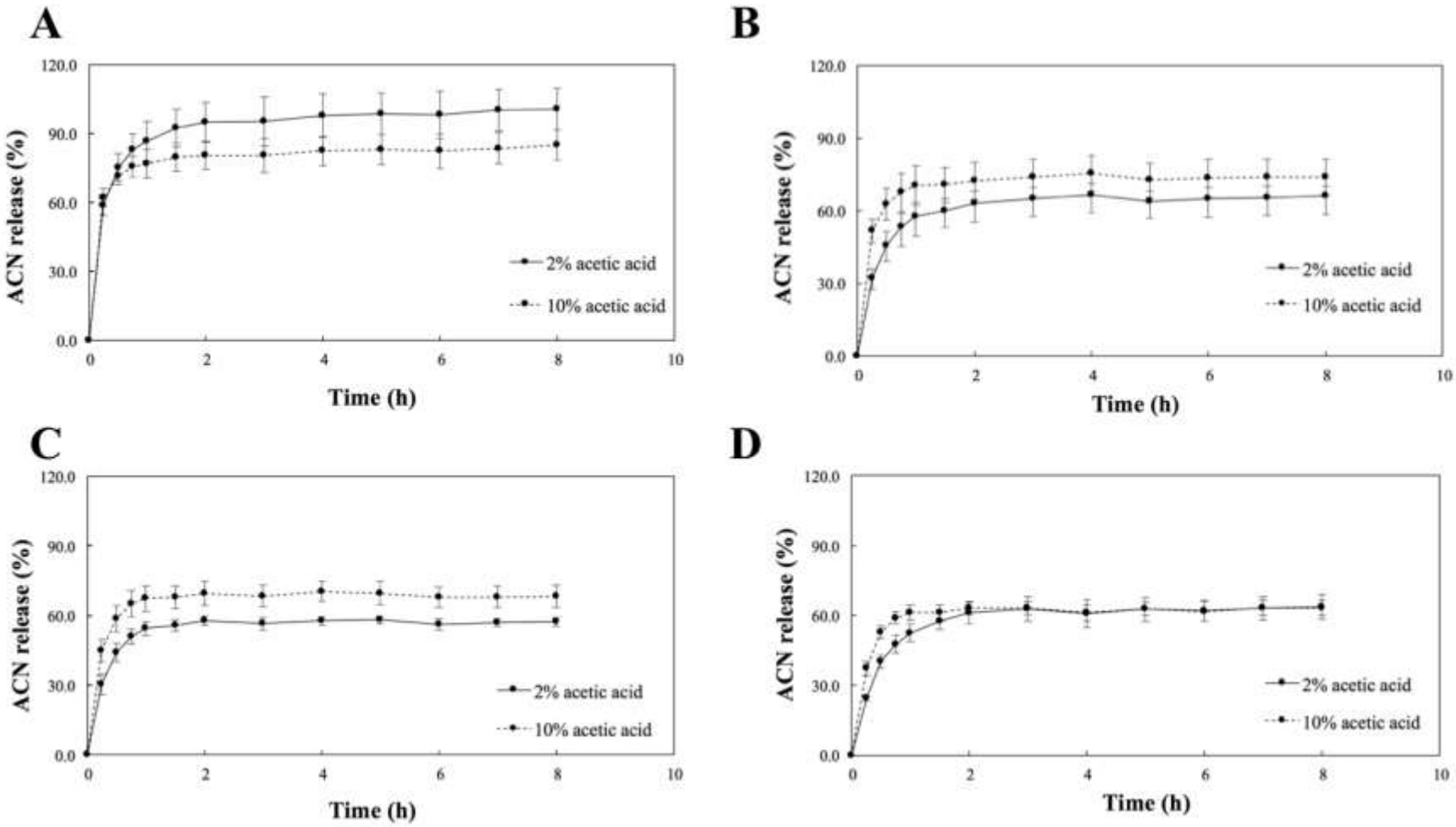

Figure 8.5 *In vitro* ACN release profile for (A) control, (B) 1:4, (C) 1:2, and (D) 3:4 $CaCO_3$:alginate ratio microspheres prepared with 2 (continuous line) and 10 % (dashed line) acetic acid in 0.1 N HCl with surfactant. Vertical bars represent the standard deviation of the measurements (n = 3)

The Peppas power law equation fitted the release data well (Table 8.1). All samples exhibited regression coefficient (R^2) values above 91 %, small standard errors of regression (S), and release exponent (n) below 0.43, indicating that the release of ACNs from the microspheres is controlled most likely by diffusion (Hodsdon et al., 1995; Peppas, Slaughter, & Kanzelberger, 2012).

A comparison of the similarity and difference factors is shown in Table 8.1. The similarity factor (f_2) is a measure of the agreement between curves and varies from 0 to 100 (Shah et al., 1998), with higher values indicating more similarity. In general, f_2 between 50 and 100 indicates a difference between the profiles equal or smaller than 10 %. The release curves showed a difference ≤10% for the following pairs: [2 %_1:4, 2 %_1:2], [2 %_1:4, 2 %_3:4], [2 %_1:2, 2 %_3:4], [10 %_1:4, 10 %_1:2], [10 %_1:2, 10 %_3:4], [2 %_1:4, 10 %_3:4], and [2 %_3:4, 10 %_3:4].

8.5 DISCUSSION

Researchers have shown that the carriers involved in ACN uptake *in vivo* can be saturated with the administration of high concentrations of these compounds, with a considerable reduction in the amount absorbed (Kurilich et al., 2005; Talavéra et al., 2003). This is the first time that floating microspheres are proposed as potential GRS for the delivery of ACNs. It is expected that the floating microspheres should be able to sustain the release of ACNs over time to prevent this saturation and overcome losses due to gastric emptying.

Alginate gels have been commonly formed by ionotropic gelation with divalent calcium ions (Mørch, Donati, Strand, & Skjåk-Bræk, 2006), by either internal or external cross-linking (Chan, Lee, & Heng, 2006). External gelation has been preferred for encapsulation purposes as it generally produces particles with smoother surface and greater strength and stiffness (Chan et al., 2006). This method has been used for the encapsulation of cocoa extract (Lupo, Maestro, Gutiérrez, & González, 2015), pomegranate peel extract (Zam, Bashour, Abdelwahed, & Khayata, 2014), thyme aqueous extract (Stojanovic et al., 2012), among others.

Table 8.1 Release kinetics data fitted by the power law, similarity (f_2) and difference (f_1) factors

Sample	2 % acetic acid				10 % acetic acid			
	Control	**1:4**	**1:2**	**3:4**	**Control**	**1:4**	**1:2**	**3:4**
POWER LAW								
R^2	0.9711	0.9330	0.9191	0.9142	0.9895	0.9723	0.9457	0.9313
S	4.93	4.95	4.95	5.85	2.42	3.55	4.73	4.91
k	0.82	0.53	0.49	0.48	0.75	0.66	0.62	0.55
n	0.12	0.14	0.11	0.17	0.07	0.07	0.07	0.08
REFERENCE								
2 % acetic acid								
Control	**100**	25.13	21.35	22.63	44.23	33.86	29.80	25.09
	0	34.76	41.15	39.09	13.47	22.36	27.30	34.31
1:4	25.13	**100**	59.43	67.49	34.77	46.39	55.32	69.96
	53.29	**0**	9.79	6.63	33.57	19.01	11.44	5.60
1:2	21.35	59.43	**100**	66.15	29.56	38.40	45.01	59.70
	69.92	10.85	**0**	8.23	48.07	31.93	23.53	11.62
3:4	22.63	67.49	66.15	**100**	30.71	39.72	46.08	58.22
	64.17	7.10	7.95	**0**	43.06	27.46	19.35	8.00
10 % acetic acid								
Control	44.23	34.77	29.56	30.71	**100**	52.89	43.86	35.47
	15.46	25.14	32.46	30.10	**0**	10.90	16.57	24.62
1:4	33.86	46.39	38.40	39.72	52.89	**100**	66.04	48.00
	28.80	15.97	24.20	21.54	12.24	**0**	6.36	15.39
1:2	29.80	55.32	45.01	46.08	43.86	66.04	**100**	59.34
	37.56	10.26	19.05	16.21	19.87	6.80	**0**	9.64
3:4	25.09	69.96	59.70	58.22	35.47	48.00	59.34	**100**
	52.23	5.56	10.41	7.42	32.66	18.19	10.67	**0**

Note: R^2 – regression coefficient; S – standard error of the regression; k – kinetic constant; n – release exponent; f_2 and f_1 are indicated in the first and second lines of each cell, respectively

In this study, $CaCO_3$ was used as the gas-generating compound for the preparation of floating microspheres, where upon extrusion of the alginate solution into an acidic gelation medium, the insoluble calcium carbonate reacts with acetic acid to produce CO_2 gas. Two concentrations of glacial acetic acid were investigated in this study (2 and 10 %, v/v) to determine which was more effective on generating gas from the carbonate.

In general, the size of fresh particles reduced by half after drying. The size of dry particles was significantly affected by the concentration of $CaCO_3$ in comparison with control samples, corroborating the results obtained by Choi et al. (2002). In addition, the size was also affected by the concentration of acetic acid used in the gelation medium, i.e. increasing the concentration of acid (10 %) resulted in significantly ($p < 0.05$) larger particles, possibly because the generation of gas was more efficient than at 2 %.

The floating capacity provides further evidence that the higher concentration of acid released more gas. With the exception of the control, a significantly higher floating capacity was observed for the samples prepared with 10 % acetic acid. For these samples, it is interesting to note that the floating capacity decreased with the increase in $CaCO_3$ concentration. It is possible that the gelation time was not sufficient for all of the added carbonate to react with acetic acid and produce gas. Choi et al. (2002) also observed that fresh particles have a different floating capacity than dry ones as they can contain more gas. In this study, it was observed that during preparation, increasing the concentration of $CaCO_3$ resulted in nearly 100 % floating capacity as more gas was produced (Ma et al., 2008), which suggests that gas could have permeated out of the microspheres during drying.

The acid concentration in the gelation bath also impacted the *EE*. A lower *EE* was found for control samples prepared with 10 % than with 2 % acetic acid. The more acidic conditions found in the gelation medium containing 10 % acetic acid could have led to a rapid precipitation of alginate as alginic acid, possibly resulting in a more porous shell than the one produced with lower concentration of acid (Hodsdon, Mitchell, Davies, & Melia, 1995). The *EE* results reported in this study are lower than previously described (Shishu et al., 2007; Nayak, Jain, & Pandey, 2011; Singh, Deol, & Kaur, 2012), however significantly higher than those reported by Ma et al. (2008). For floating microspheres, it is possible that the calcium ions released from the salt could have contributed to the

internal gelation process as higher *EE* were observed at higher concentrations of carbonate.

In relation to release, Choi et al. (2002) showed that increased concentrations of $CaCO_3$ resulted in prolonged release of riboflavin from alginate particles, possibly due to the internal gelation promoted by calcium ions. For samples prepared in both gelation media, increasing the concentration of carbonate resulted in lower amounts of ACN released. In order to investigate the release mechanism, data were initially fitted to first-order kinetics, Higuchi law, and Peppas power law equations. However, due to the considerably low regression coefficients (R^2), first-order kinetics and Higuchi law were not considered for further analysis. The Peppas power law equation was a good fit for the experimental data, with R^2 ranging from 91.91 to 98.95 %. The release exponent (n) below 0.43 suggested that the release was most likely controlled by diffusion (Hodsdon et al., 1995; Peppas et al., 2012), in agreement with Singh et al. (2010) and Zhang et al. (2011).

The release curves were compared using the similarity (f_2) and difference (f_1) factors as a measure of agreement between them. The f_2 factor varies from 0 to 100 (Shah et al., 1998), with higher values indicating more similarity. In general, f_2 values between 50 and 100 indicate a difference (f_1) between the profiles that is equal or smaller than 10 %. Considering the release curves that showed a difference ≤10 %, particles with a 1:4 ratio prepared with 2 % acetic acid exhibited a similar profile to particles with the other concentrations of carbonate. In the case of experiments using the 10 % acetic acid, the release curve for the particles with a 1:4 ratio was similar to the curve for the 1:2 ratio, whereas the curve for the 1:2 ratio was similar to that for the 3:4 ratio.

Taking all of the data into consideration, the sample prepared with 10 % acetic acid and 1:4 $CaCO_3$:alginate ratio displayed the best results in comparison to the other samples. Although the size and *EE* were slightly lower than at 1:2 and 3:4 ratios, the floatability of dry particles was better, possibly because higher concentrations of carbonate could have burst the microspheres during gelation.

8.6 CONCLUSION

In this study, floating alginate microspheres were prepared by ionotropic gelation method for the oral delivery of ACNs extracted from haskap berries, using $CaCO_3$ as gas-generating compound. It was found that the inclusion of carbonate resulted in larger particles, higher *EE*, and lower concentrations of ACNs released over time. The floatation capacity of the microspheres was affected by the concentration of acetic acid used in the gelation media, i.e. higher concentration improved floatation. The Peppas power law equation fitted the data well, suggesting that the main mechanism of release was most likely diffusion. Floating microspheres produced with 10 % acetic acid could be used as GRS for the delivery of ACNs in their absorption window, especially with a 1:4 $CaCO_3$:alginate ratio.

Chapter 9 DEVELOPMENT AND EVALUATION OF A NOVEL *IN SITU* SYSTEM FOR MODULATION OF THE RELEASE OF ANTHOCYANINS FROM HASKAP BERRIES (*LONICERA CAERULEA* L.)

Materials of this chapter have been submitted for publication in **Food Hydrocolloids**.

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9.1 ABSTRACT

Several health benefits have been associated with the consumption of anthocyanins (ACNs), an important group of natural hydrophilic pigments. These compounds are absorbed in the upper digestive tract, namely in the stomach and initial sections of the small intestine. However, an insufficient residence time of ACNs in these organs could result in limited absorption and contribute to degradation and thus, gastroretentive systems could be a useful strategy to increase the retention time in their absorption sites. In this paper, a novel *in situ* gelling system is described as a platform to modulate the release and increase the retention time of ACNs from haskap berries in sites where their absorption and stability are favored. Thirteen formulations were assessed, where their composition differed by the concentrations of alginate, sodium bicarbonate, and calcium carbonate. Freeze-dried ACN-rich extract from haskap berries was incorporated into selected formulations. The ACN release profile indicated that the Peppas (power law) equation had the best fit to the experimental data and diffusion was most likely the dominant release mechanism.

9.2 INTRODUCTION

Anthocyanins (ACNs) are an important group of hydrophilic pigments in higher plants (Delgado-Vargas et al., 2000). These compounds have been associated with reduced risk of degenerative diseases (Cassidy et al., 2011, 2013; Pascual-Teresa et al.,

2010; Wallace, 2011; Wang & Stoner, 2008), including type 2 diabetes (T2D) (Guo & Ling, 2015; van Dam et al., 2013). For instance, recent reports have indicated that ACNs from haskap berries (*Lonicera caerulea* L.) could contribute to the management of T2D by controlling weight gain (Wu et al., 2013) and reducing postprandial blood glucose levels (Takahashi et al., 2014). Due to the high levels of ACNs in these fruits, they could be used as a source of bioactive compounds for the development of value-added products with health-promoting benefits (Celli et al., 2014).

Research has shown that ACNs are absorbed in the upper gastrointestinal (GI) tract (stomach and initial part of the small intestine) (Felgines et al., 2007; Passamonti et al., 2003). An insufficient residence time of ACNs in these organs could result in limited absorption and contribute to degradation at high pHs found in the intestines (Liu et al., 2014; McDougall et al., 2005) and due to metabolism (Czank et al., 2013; Ferrars et al., 2014; Fleschhut et al., 2006; Kay et al., 2005). In order to modulate the release and absorption of ACNs, the use of gastroretentive systems (GRS) could be a promising strategy to increase their retention time in parts of the GI tract where they are absorbed (Celli, Kalt, & Brooks, 2016). Among the different GRS platforms that could be used by the food industry (Sharma & Khan, 2014), intra-gastric floating *in situ* gelling systems (or raft systems) are advantageous as the product could be commercialized in a powder form and reconstituted immediately before consumption, in a simple user-controlled preparation.

In situ gelling systems are often used for the treatment and amelioration of heartburn symptoms and as reflux suppressants (Ibrahim, 2009; Rajinikanth, Balasubramaniam, & Mishra, 2007; Rao & Shelar, 2015; Strugala, Dettmar, & Thomas, 2012; Tytgat & Simoneau, 2006; Zentilin et al., 2005). More recently, these systems have been proposed as drug delivery platforms (Prajapati, Mehta, Modhia, & Patel, 2012; Rajinikanth et al., 2007). They consist of polymers (e.g. alginates) that gel when in contact with body fluids or due to changes in pH (Kubo et al., 2004), combined with gas-generating compounds (e.g. carbonate). Alginates (p*K*a 3.4-3.65) often require a divalent cation for the sol to gel transformation or low gastric pH to precipitate as alginic acid (Hampson et al., 2010). The gas released is entrapped in the gel matrix (forming a foamy gel structure), which reduces the density of the system and contributes to its flotation

above the gastric content (Tang et al., 2010). Reports have indicated that these gels can remain in the stomach for longer periods of time in comparison to a meal (Davies, Farr, Kellaway, Taylor, & Thomas, 1994; Dettmar, Little, & Baxter, 2005; Strugala et al., 2012; Washington, Greaves, & Wilson, 1990) and increase the efficiency of drugs that are absorbed and/or act locally (Chevrel, 1980; Rajinikanth et al., 2007).

To the best of our knowledge, this is the first time that an *in situ* gelling system is described as a platform for the delivery of ACNs. Thus, the objective of the present study was to develop an *in situ* gelling formulation consisting of ACN-rich extract from haskap berries and determine its characteristics, such as the gelling capacity, gel weight, volume, density, thickness, resilience, and release profile. Thirteen base formulations were initially assessed and their composition differed by the concentrations of sodium alginate, sodium bicarbonate, and calcium carbonate. The best formulations were selected based on their gelling capacity and resilience at 0.1 and 0.05 N HCl. From this, freeze-dried fractionated haskap berry extract was incorporated and the ACN release profile under acidic conditions was measured over time.

9.3 MATERIAL AND METHODS

9.3.1 Materials

Formic and hydrochloric acids, reagent ethanol, calcium carbonate, potassium chloride, and sodium acetate anhydrous were purchased from Fisher Scientific (Ottawa, ON, Canada). Low-viscosity sodium alginate Protanal LFR5/60 (65–75 % guluronic acid) was kindly donated by FMC BioPolymer (Philadelphia, PA, USA). Sodium bicarbonate was acquired from Church & Dwight Co., Inc. (Princeton, NJ, USA).

9.3.2 Plant Material

Haskap berries (var. *Indigo Gem*) grown in Saskatchewan, Canada, were used in this study. Berries were harvested, packed, and shipped frozen to Nova Scotia. The berries were kept at –35 ºC prior to chopping in half and freeze-drying in a Labconco FreeZone 2.5 L Bench-top Freeze Dry System (Labconco, Kansas City, MO, USA) until

the final moisture content was below 5 % (w/w). The freeze-dried samples were stored in a desiccator at –18 ºC until use.

9.3.3 Preparation of Anthocyanin-Rich Freeze-Dried Haskap Extract

The extraction of ACNs from freeze-dried haskap berries was conducted according to Celli et al. (2015c). Briefly, 80 % ethanol acidified with 0.5 % formic acid was added to freeze-dried samples in a solid to solvent ratio of 1:25 (w/v) and stirred at 600 rpm for 10 min at room temperature using a magnetic stirrer. Then, the mixture was filtered through a Whatman filter paper no. 1 and the extract was kept at –18 ºC in the absence of light until further use.

Prior to fractionation, the organic solvent was removed using a rotary evaporator model RE-51 (Yamato Scientific America Inc., Santa Clara, CA, USA) kept at $T < 50$ ºC, equipped with a vacuum pump V-700 (Büchi Labortechnik AG, Flawal, Switzerland) and a water circulation cooler WKL 230 (Lauda, Lauda-Königshofen, Germany). The resultant fraction was kept at –18 ºC and protected from light until further use.

The fractionation of the evaporated extract was performed according to Kalt et al. (2008b) with some modifications. A column consisting of approximately 300 g of C18 bulk packing material (Waters, Missisauga, ON, Canada) was previously washed with water. The evaporated extract was added to the column and washed with approximately two volumes of water to remove sugars. The bound components were eluted with 1.5 volume of reagent ethanol (acidified with 0.5 % formic acid). The solvent of the resultant fraction was removed by rotary evaporation and freeze drying, and the powder was stored at –18 ºC prior to use.

9.3.4 Preparation of *In Situ* Gelling System

The *in situ* gelling base formulations investigated in this study are presented in Table 9.1 and consisted of different concentrations of sodium alginate (x_1, 1.5 to 3.5 %, w/v), sodium bicarbonate (x_2, 1.5 to 2.5 %, w/v), and calcium carbonate (x_3, 0.5 to 2 %, w/v). The range of concentrations was determined in preliminary experiments, which

indicated that these formulations could be easily dispersed and gelled within seconds when in contact with acidic solutions. The powder mixtures were dissolved/dispersed with distilled water immediately prior to use by vigorous manual agitation for 30 s in a Falcon tube at room temperature, to simulate preparation by the consumer, and further evaluated for their properties. Gel characteristics were evaluated (gelling capacity, weight, volume, density, thickness, and resilience) and the most appropriate formulations were selected for further incorporation of the ACN-rich powdered extract.

9.3.5 *In Vitro* Evaluation of the *In Situ* Gelling System

9.3.5.1 Gelling Capacity (Raft Formation)

The raft-forming ability of the formulations (as measured by assessing the gelling capacity of each formulation) was evaluated according to the method described by Rao and Shelar (2015). Briefly, 5 mL of the gelation solution consisting of 0.1 N or 0.05 N HCl was transferred to a 15-mL borosilicate tube and maintained at 37 ± 0.5 ºC in a water bath. Then, 1 mL of the gelling solution was added by placing the pipette tip at the surface of the gelation solution and slowly releasing its contents into the test tube. The formulations were assessed visually for the time required to complete the gelation and any dispersion of the gel up to 24 h after preparation.

Table 9.1 Composition of the formulations investigated in this study and experimental results

Form.	x_1 (%)	x_2 (%)	x_3 (%)	0.1 N HCl					0.05 N HCl				
				Weight (g)	Volume (mL)	Density (g/mL)	Thickness (mm)	Resilience (min)	Weight (g)	Volume (mL)	Density (g/mL)	Thickness (mm)	Resilience (min)
F1	1.5	1.5	1.25	12.0±0.2	21.2±0.8	0.56±0.02	9.5±0.1	5-10	-	-	-	-	-
F2	3.5	1.5	1.25	22.2±0.8	36.5±0.5	0.61±0.03	12.6±0.1	>120	32.9±1.9*	35.5±2.2	0.93±0.02*	16.2±0.1*	>120
F3	1.5	2.5	1.25	9.6±0.4	16.5±1.1	0.58±0.02	8.7±0.1	5-10	-	-	-	-	-
F4	3.5	2.5	1.25	25.7±0.4	55.1±0.5	0.47±0.01	18.6±0.3	>120	42.9±1.7*	55.3±1.0	0.78±0.03*	19.6±0.2*	>120
F5	1.5	2	0.5	11.5±0.3	18.4±1.1	0.63±0.02	8.8±0.2	2-5	-	-	-	-	-
F6	3.5	2	0.5	22.1±0.2	35.9±1.2	0.62±0.02	10.5±0.3	>120	-	-	-	-	-
F7	1.5	2	2	12.8±0.1	30.0±1.4	0.43±0.02	9.8±0.4	30-45	-	-	-	-	-
F8	3.5	2	2	25.1±0.3	47.3±1.1	0.53±0.02	14.7±0.3	>120	39.3±0.6*	48.8±0.3	0.80±0.02*	16.6±0.1*	>120
F9	2.5	1.5	0.5	17.8±0.5	23.3±0.9	0.76±0.03	10.5±0.2	10-20	-	-	-	-	-
F10	2.5	2.5	0.5	18.0±0.7	34.8±2.2	0.52±0.05	10.1±0.9	>120	-	-	-	-	-
F11	2.5	1.5	2	19.8±0.1	29.9±1.2	0.66±0.03	8.5±3.5	>120	30.0±2.1*	32.9±1.3	0.91±0.03*	14.1±0.1*	105-120
F12	2.5	2.5	2	21.5±1.5	49.4±1.2	0.44±0.04	16.3±2.1	>120	33.0±2.0*	49.0±1.1	0.67±0.03*	15.9±0.3	45-60
F13	2.5	2	1.25	19.8±0.2	32.6±0.4	0.61±0.01	11.5±1.1	>120	31.1±1.5*	42.5±2.4*	0.73±0.01*	15.6±0.1*	>120

Note: x_1 – sodium alginate concentration (%); x_2 – sodium bicarbonate concentration (%); x_3 – calcium carbonate concentration (%).

*Means for weight, volume, density, and thickness at 0.1 and 0.05 N HCl are significantly different at $p < 0.05$, according to the paired *t*-test

9.3.5.2 Raft Volume, Weight, Density, and Thickness

Raft weight and volume were determined according to the procedure by Hampson et al. (2005) and used to calculate the density and measure the thickness of the gels formed. Briefly, each beaker used for raft formation was pre-weighed (**W1**). Then, 10 mL of the formulation were added to 150 mL of 0.1 N or 0.05 N HCl maintained at 37 ± 0.5 ºC and the gel was allowed to develop for 30 min. The outside of the beaker was marked at the position to which the top of the gel reached and the beaker (and its contents) weighed (**W2**). The subnatant liquid was carefully decanted. The gel was left to stand for 30 s in the beaker, the excess liquid was drained off, and the gel was weighed (**W3**). The last step consisted of removing any liquid from the inside of the beaker, refilling with water to the marked position, and weighing (**W4**). For this experiment, it was assumed that the density of the gelation solution was similar to the water (i.e. 1 g/mL). All weights were expressed in g. The raft volume was calculated as follows:

$$Raft\ volume\ (mL) = (W4 - W1) - (W2 - W1 - W3) \quad (41)$$

The raft density was calculated by dividing the raft weight (in g) by its volume (in mL). Each gel was measured in three places using a caliper ruler to determine gel thickness and account for differences in gel formation (Johnson, Craig, Mercer, & Chauhan, 1997), and the average was reported.

9.3.5.3 Raft Resilience

Raft resilience was assessed using the procedure by Hampson et al. (2005, 2010) slightly modified. Briefly, 10 mL of the formulation were added to 150 mL of 0.1 N or 0.05 N HCl in a 250-mL glass jar, maintained at 37 ± 0.5 ºC in a reciprocal shaker bath Model 2870 (Thermo Scientific, Marietta, OH, USA). The gels were developed for 30 min, undisturbed. Then, the jars were closed and placed in the shaker at 95 cycles per min. The gels were visually assessed for size and coherence after 2, 5, 10, 20, 30, 45, 60 min, and up to 120 min. Raft resilience was defined as the last time point at which a

floating gel of at least 15 mm in diameter was observed. Raft resilience was initially assessed using 0.1 N HCl. The formulations that resisted for a longer period of time under agitation were evaluated for their gelling properties and resilience using 0.05 N HCl.

9.3.5.4 Anthocyanin Loading Capacity

The formulations that exhibited the highest resilience using 0.05 N HCl were selected for incorporation of the ACN-rich freeze-dried extract (321.96 ± 8.35 mg cyanidin 3-glucoside equivalents per g of freeze-dried extract). Different amounts of freeze-dried extract were incorporated, namely 1 and 5 mg of freeze-dried extract per mL of gelling solution. The loading capacity of the gelling solutions was assessed by the method described by Rao and Shelar (2015), slightly modified. Briefly, 3 mL of the gelling solution was added to a 200-mL volumetric flask, followed by the addition of 150 mL of 0.1 N HCl. The mixture was shaken in a Series 25 Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm for 40 min, the volume was made up to 200 mL, followed by sonication for 30 min in an ultrasound bath Branson 2510R-DTH (Bransonic, Danbury, CT, USA). The final solution was assessed for its total ACN content (TAC) and compared to a control consisting of the same concentration of freeze-dried extract in 0.1 N HCl.

9.3.6 Measurement of *In Vitro* Anthocyanin Release

The release of ACNs from the *in situ* gels was determined by a semi-dynamic method in reciprocal shaker bath Model 2870 (Thermo Scientific) maintained at 37 ± 0.5 ºC, undisturbed. The release medium consisted of 150 mL of 0.1 N HCl in a 250-mL beaker. This volume was similar to that used to characterize the formulations and is in the range of gastric emptying rate (on average 2–4 mL/min) (Koziolek, Garbacz, Neumann, & Weitschies, 2013) and volume during the fasted state (Fidler et al., 2009). A 10-mL aliquot of the *in situ* gelling solution was pipetted into the HCl solution, with an adapted plunger and mesh screen in position (Figure 9.1). At each time interval (0.5, 1, and hourly up to 8 h), the plunger was removed with the raft on the screen and placed in a fresh

medium (150 mL HCl), pre-warmed (37 ºC). The first sample was removed after 30 min to evaluate the release during gel formation and settlement. The acid solutions containing ACNs were assessed for their TAC.

ACN release kinetics were analyzed by plotting the mean release data *vs.* time. SigmaPlot™ version 13.0 software (Systat Software, Inc., Sao Jose, CA, USA) was used to fit the curves for first-order kinetics, Higuchi law (Higuchi, 1961), and Peppas equation (or power law) (Ritger & Peppas, 1987; Siepmann & Peppas, 2001), expressed as follows:

First-order kinetics: $\frac{M_t}{M_\infty} = 100(1 - e^{-kt})$ (42)

Higuchi law: : $\frac{M_t}{M_\infty} = k\sqrt{t}$ (43)

Peppas equation: : $\frac{M_t}{M_\infty} = kt^n$ (44)

where M_t and M_∞ are the cumulative absolute amount of ACNs released at time t and infinite time, respectively; k is the kinetic constant; and n is the release exponent that characterizes the diffusional mechanism.

9.3.7 Total Anthocyanin Content (TAC)

The TAC was determined by the pH-differential method described by Giusti and Wrolstad (2001). Absorbances were measured at λ_{max} and 700 nm in a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Madison, WI, USA) against distilled water.

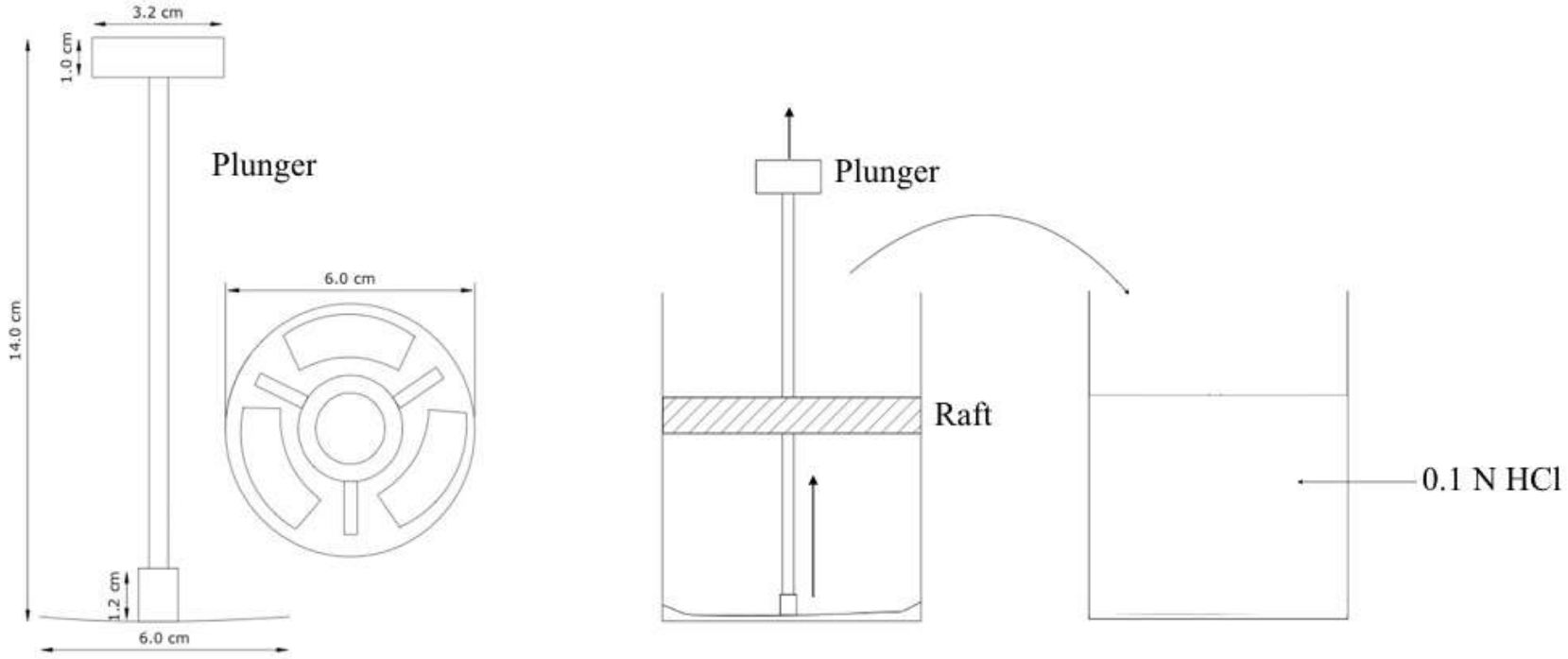

Figure 9.1 Schematic representation of the plunger used to transfer the gel to fresh HCl solution (left) and the semi-dynamic procedure used to determine the release profile (right)

9.3.8 Statistical Analysis

All the experiments were conducted in triplicate and results were reported as mean ± standard deviation. Results for raft weight and volume formed at 0.1 N HCl were analyzed by multiple regression using Minitab® version 17.1.0 software (Minitab Inc., State College, PA, USA) to evaluate the statistically significant predictors and their contribution to weight and volume. For this analysis, the predictors were standardized by subtracting the mean to remove multicollinearity produced by interaction and higher-order terms. Pearson's correlation analyses of thickness *vs*. weight and volume was also performed. Weight, volume, density, and thickness of gels prepared with 0.1 and 0.05 N HCl were compared by paired *t*-test to assess the effect of acid concentration on these properties.

9.4 RESULTS AND DISCUSSION

Researchers have indicated that the consumption of ACNs is positively associated with reduced risk and management of T2D (Hoggard et al., 2013; Muraki et al., 2013; Samieri et al., 2014; Stull et al., 2010; Törrönen et al., 2010, 2012a, 2012b; Wedick et al., 2012). ACNs are generally stable in the stomach (Liang et al., 2012), which acts as an absorption site for these compounds via the bilitranslocase carrier (Felgines et al., 2007; Passamonti et al., 2003). Studies have indicated that the peak of plasma ACN concentration is achieved within 0.5–2 h after ingestion (McGhie & Walton, 2007), which coincides with the gastric emptying time. In addition, it has also been shown that reducing the gastric emptying rate by the concomitant consumption of phytic acid (Matsumoto et al., 2007) or cream (Mullen et al., 2008) can result in increased and delayed absorption of ACNs, respectively, corroborating the hypothesis that the stomach plays an important role in the absorption of these compounds. ACNs that are not absorbed in the upper GI tract are subjected to extensive metabolism (Czank et al., 2013; Fleschhut et al., 2006) and degradation due to changes in pH (McDougall et al., 2005). These modifications to the ACN native structure would limit the amount of the parent glycoside available to result in a positive biological effect.

In order to increase the retention time of the ACNs in sites of the GI tract where their absorption and stability are favored, a novel *in situ* gelling system was developed. Thirteen base formulations with varying concentrations of sodium alginate, sodium bicarbonate, and calcium carbonate were evaluated. The next sections discuss the characteristics of the different base formulations and the incorporation of ACN-rich freeze-dried haskap berry extract to assess the release profile.

9.4.1 Characteristics of the *In Situ* Gelling Formulations

The composition of the thirteen formulations investigated is presented in Table 9.1. The viscosity of the alginate solution does not seem to play a role in the gelling process; however, high viscosities can be detrimental to the ease of swallowing the formulation and acceptance by the consumer (Ibrahim, 2009). A visual assessment of the formulations indicated that they were fluid upon preparation and would not cause any issue for swallowing. Johnson et al. (1997) showed that the low molecular weight alginate LFR5/60 used in this study produced gels with larger volume and thickness than other alginates.

One of the pre-requisites of *in situ* intra-gastric floating gelling systems is the gelling capacity (Rajinikanth et al., 2007). All the formulations investigated in this study exhibited a high gelling capacity at 0.1 N HCl maintained at 37 ºC, i.e. gelled instantaneously when in contact with the acidic medium and retained the gel structure for more than 24 h undisturbed. Hampson et al. (2005) have shown that commercial *in situ* gelling anti-acid formulations that contain alginate, bicarbonate, and carbonate often form gels immediately when in contact with HCl solution and exhibit complete flotation on the surface of the liquid and good coherence (i.e. the gel is held together as one mass when manipulated). This study corroborated our results and indicated that all formulations were appropriate for further investigation.

Raft weight and volume were determined after gel formation and stabilization (t = 30 min). Excess subnatant was carefully removed to prevent any disturbance in the integrity of the gels. Multiple regression analyses showed that the concentrations of alginate (x_1), bicarbonate (x_2), and carbonate (x_3) significantly affected the weight and volume of the gels formed in 0.1 N HCl at 37 ºC, in addition to some quadratic and

interaction terms (Table 9.2). Some examples of the gels formed are presented in Figure 9.2. The significant interaction of alginate with calcium and bicarbonate salts on the weight could be explained by the mechanism of gel formation and flotation, which requires calcium ions in solution and gas (namely CO_2 gas), respectively. In addition, the gel most likely entrapped liquid during its formation, which could have contributed to the weight of the gel. Initially, the gelling suspension sinks to the bottom of the vessel due to higher density until it has sufficient time to react with the acid (in this study, within seconds) and float (Hampson et al., 2010). For example, comparing F5 with F7 and F9 with F11, there was an increase in the gel weight when increasing the calcium carbonate concentration, keeping the concentrations of alginate and bicarbonate the same. Increasing the concentration of bicarbonate also contributed to the weight, as in the cases of F9 compared with F10 and F2 with F4. The polynomial equation for weight was as follows:

$$Weight\ (g) = -0.55 + 9.17x_1 + 0.55x_2 - 1.78x_3 - 1.92x_1^2 - 2.09x_2^2 + 2.93x_1x_2 + 0.57x_1x_3 + 1.00x_2x_3 \quad (45)$$

In the case of volume, the interaction terms x_1x_2 and x_2x_3 were also significant ($p < 0.05$), which indicates the need of a polymeric matrix to maintain the gel structure and entrap the gas formed during acidification of the gelling solution. Similar to weight, increasing the concentration of bicarbonate (keeping the other variables constant) resulted in a larger gel volume, such as in the case of the pairs F9–F10 and F2–F4. The same trend was observed when the concentrations of alginate and carbonate increased, for example, in F5–F6 and F10–F12, respectively. The volume equation was found to be as follows:

$$Volume\ (mL) = 45.2 - 12.14x_1 - 24.5x_2 - 3.33x_3 + 11.62x_1x_2 + 5.36x_2x_3 \quad (46)$$

Table 9.2 Multiple regression analysis for gel weight and volume as responses

Source	*df*	SS	MS	*F*-value	*p*-value
Weight					
Regression	8	1001.96	125.246	278.84	<0.001
x_1	1	901.60	901.60	2007.26	<0.001
x_2	1	3.60	3.604	8.02	0.008
x_3	1	36.02	36.02	80.18	<0.001
x_1^2	1	31.05	31.05	69.13	<0.001
x_2^2	1	2.29	2.29	5.11	0.031
x_1x_2	1	25.81	25.81	57.47	<0.001
x_1x_3	1	2.17	2.17	4.83	0.036
x_2x_3	1	1.69	1.69	3.76	0.062
Error	30	13.48	0.449		
Total	38	1015.44			
S	0.67		Adjusted R^2	0.9832	
R^2	0.9867		Predicted R^2	0.9772	
Volume					
Regression	5	4897.80	979.559	104.84	<0.001
x_1	1	2952.60	2952.60	316.02	<0.001
x_2	1	757.13	757.13	81.04	<0.001
x_3	1	734.83	734.83	78.65	<0.001
x_1x_2	1	404.84	404.84	43.33	<0.001
x_2x_3	1	48.40	48.40	5.18	0.029
Error	33	308.32	9.343		
Total	38	5206.12			
S	3.06		Adjusted R^2	0.9318	
R^2	0.9408		Predicted R^2	0.9136	

Note: R^2 – regression coefficient; *S* – standard error of the regression

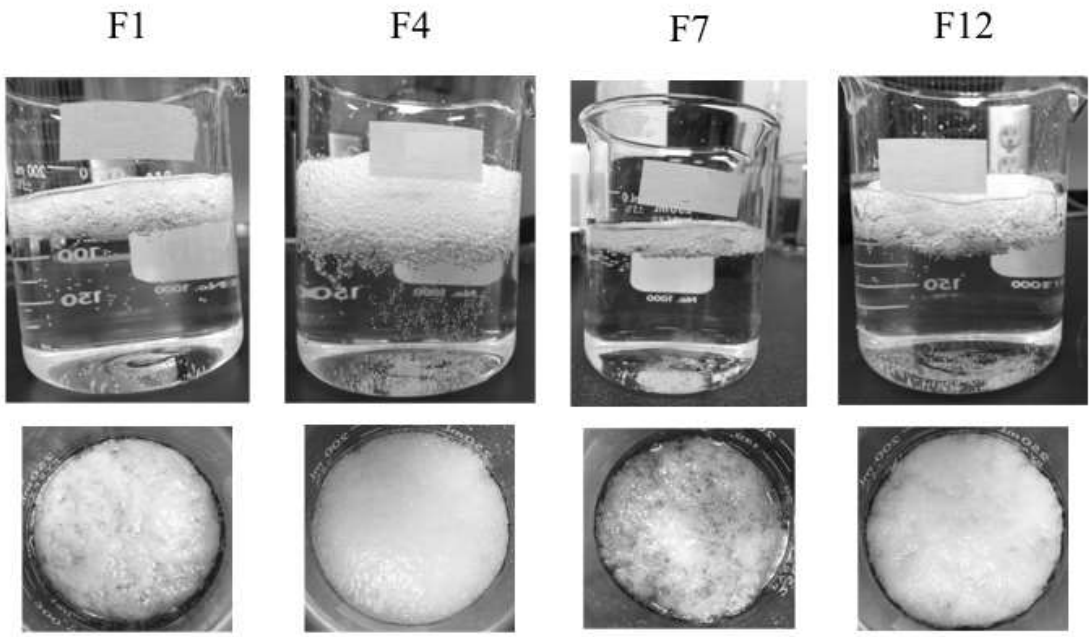

Figure 9.2 Examples of the gels formed at 0.1 N HCl during weight and volume assessment

The density of the gels, calculated by dividing the weight (in g) by its volume (mL), varied greatly among the formulations (Table 9.1). Holding the concentrations of alginate and bicarbonate constant, an increase in carbonate concentration resulted in a reduction of the density, such as for the pairs F5–F7, F9–F11, and F6–F8. Similarly, the density decreased when increasing the bicarbonate concentration (setting the other concentrations constant), for example, in F2–F4, F9–F10, and F11–F12. Carbonate and bicarbonate release gas under acidic conditions, which would contribute to the reduced density of these gels.

The Pearson's correlation coefficient (r) was then calculated in order to determine the contribution of raft weight and volume on its thickness. A highly positive relationship was found for thickness and weight ($r = 0.751$, $p < 0.001$), and thickness and volume ($r = 0.902$, $p < 0.001$). Since the dimensions of the container, amount of acid and formulation, and time for gel formation and settlement were the same for the different formulations, a difference in thickness (or gel height) would be the result of the components used for their preparation (Kapadia & Mane, 2007). Since the gel had a limited space in the beaker to develop (radius of the container), increasing the volume would result in a thicker gel.

Raft resilience was evaluated as a measure of the durability and stability of the system under vigorous agitation, to mimic the gastric movement (Hampson et al., 2010), with further assessment by their visual appearance, coherence, and overall integrity of the gel after a certain period of time (Hampson et al., 2005) (Table 9.1). Undigested particles from ingested food and mucus residue remain in the stomach until approximately 2 h after the digested food has left this organ (Washington, Washington, & Wilson, 2001). Ideally, *in situ* gelling systems should float above the gastric content and remain in the stomach for up to 4 h (Strugala et al., 2012). For this assessment, 2 h was selected as the minimum amount of time that the gels should resist when agitated during the resilience test. It is worth noting that the resilience was improved when the weight and volume of the gel increased (Table 9.1). The formulations that exhibited highest resilience (above 2 h) at 0.1 N HCl (namely F2, F4, F6, F8, F10, F11, F12, and F13) were assessed for their physicochemical characteristics at a lower concentration of acid (0.05 N HCl).

The pH of the stomach can vary greatly during the day, and also depends on the position within the organ (Bumm & Blum, 1987). For this reason, it is important to evaluate the gelling performance of the formulation in lower concentrations of acid. The evaluation of gel capacity at 0.05 N HCl has indicated that F6 and F10 did not gel properly and were removed from further experiments. Among the formulations investigated at this stage, F6 and F10 exhibited the lowest concentration of calcium carbonate (0.5 %, w/v), which was not sufficient to hold the gel together in a less acidic environment. The volume of the gels was not significantly different ($p < 0.05$) at 0.1 and 0.05 N HCl, with the exception of F13 (Table 9.1). However, all the gels were significantly heavier ($p < 0.05$), which affected their density. There was also a significant difference in relation to the thickness of the gels (except for F12). During gelation, it is possible that the gels did not develop similarly when prepared with 0.1 and 0.05 N HCl. The increased thickness of gels made at 0.05 N HCl may be an indication that more liquid is entrapped in the gel matrix, which contributed to increases in the weight and density, but not the volume of the raft. These observations are in line with Patel (1991), who showed that the thickness of the gels decreased with increasing acid concentration (as reported by Johnson et al., 1997). Conversely, other authors suggested that the thickness

(Johnson et al., 1997) and weight (Tytgat & Simoneau, 2006) of the gels increased with increasing acid concentration.

The resilience test at 0.05 N HCl showed that F11 and F12 would not retain their gel structure for longer periods of time when compared to the other formulations and, for this reason, were excluded from further investigation. A visual assessment of the four remaining formulations (F2, F4, F8, and F13) indicated that F2 and F8 retained most of their gel structure after 2 h. In this way, they were selected as base formulations for the incorporation of the ACN-rich extract.

9.4.2 Incorporation of Anthocyanin-Rich Extract

The daily intake of ACNs in the USA was previously estimated to range between 180 and 220 mg (Kühnau, 1976), which could be an overestimation due to inappropriate analytical methods. Wu et al. (2006) updated this value to 12.5 mg/day based on the ACN concentration of different foods and data from their intake. The consumption of ACNs is generally considered to be safe (Pojer et al., 2013), possibly due to their low absorption (Martin & Appel, 2010), despite the high consumption in certain places. However, Ziberna et al. (2010) recently showed a biphasic behavior of ACNs in relation to the concentration. The acute administration of ACN-rich solutions in excised rat hearts at low concentrations (0.01–1 mg/L) has been associated with cardioprotective effects, reduction of LDH release rate and incidence, and duration of repercussion arrhythmias, whereas high concentrations (5–50 mg/L) were considered cardiotoxic (Ziberna et al., 2010).

Considering that the absorption of ACNs is expected to increase with the use of GRS, the concentrations evaluated in this study were low and within the range indicated by Wu et al. (2006) from the usual consumption through the diet. Various concentrations of freeze-dried extract were incorporated into F2 and F8 without any significant change in the floating properties of the gels. The incorporation of 1 and 5 mg of freeze dried extract per mL of formulation resulted in the overall loading of approximately 3.31 ± 0.04 and 15.98 ± 0.14 mg of cyanidin 3-glucoside equivalents, respectively. No significant losses in ACN content were observed during dissolution/dispersion of the formulations and preparation of the gels.

9.4.3 Release Profile

Researchers have shown that increasing the concentration of ACNs can result in the saturation of the carriers involved in their uptake, with a considerable reduction in the amount absorbed (Fernandes et al., 2012; Kurilich et al., 2005; Talaverá et al., 2003). Thus, the proposed GRS should be able to sustain the release of ACNs over time to prevent the saturation of the carriers and reduce the concentrations needed to observe an effect *in vivo*. As it has been shown that the uptake of parent ACNs by bilitranslocase occurs rapidly (Talaverá et al., 2003; Vanzo et al., 2011), the goal is to control the release of ACNs over time instead of increasing their concentration.

Figure 9.3 shows the release profile of F2 and F8 with added freeze-dried haskap extract (1 and 5 mg/mL) at 0.1 N HCl. No significant difference ($p < 0.05$) was observed between the release profile of F2 and F8 containing different amounts of freeze-dried ACN-rich extract. F2 and F8 had the same concentration of sodium alginate but varied in the amount of sodium bicarbonate and calcium carbonate, which did not seem to play a major role in the release profile. Even though F2 contained a lower concentration of calcium carbonate in comparison to F8, the release profile was virtually the same. However, Rajinikanth et al. (2007) noted that increasing the concentration of calcium carbonate resulted in a reduction of the percentage of amoxicillin released from their floating systems, which was not observed for the amounts investigated in this study.

The results also indicated that there was no significant difference ($p < 0.05$) between the rate and extent of ACNs released from the gels containing different amounts of freeze-dried extracts, for example F2 with 1 and 5 mg/mL (Figure 9.3). For all formulations, the release profile was characterized by a biphasic pattern, in accordance with the results by Rajinikanth et al. (2007). During the initial phase (up to 30 min), a high amount of ACNs was released into the acid solution (burst), followed by a reduction in release rate as the gel sets.

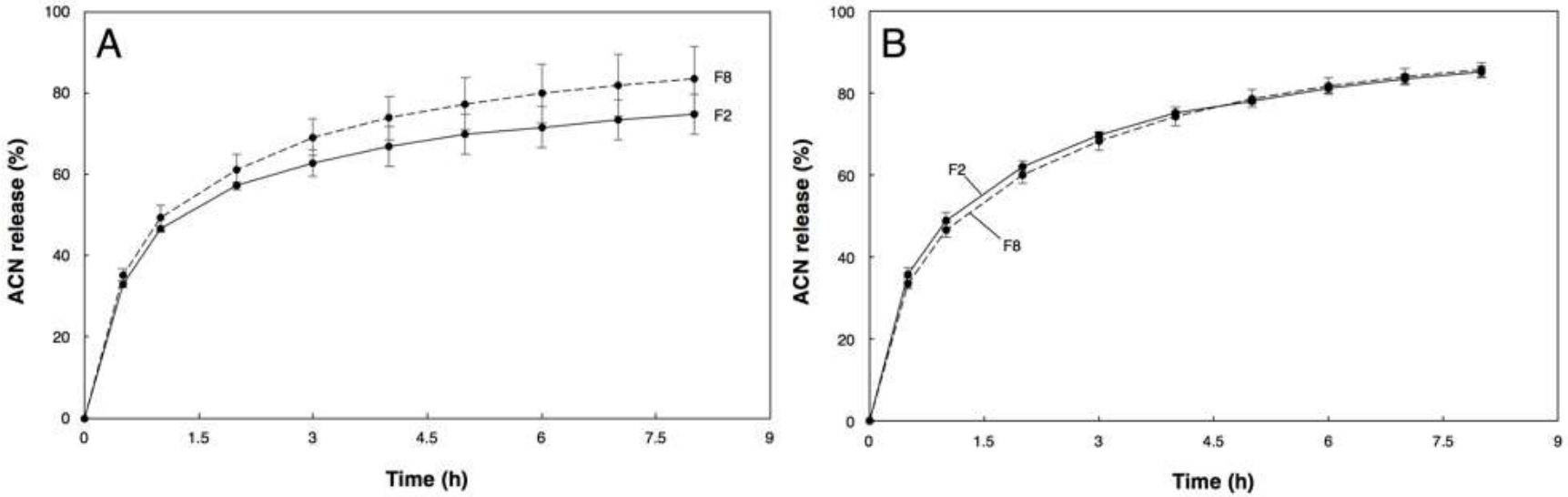

Figure 9.3 *In vitro* ACN release profile from F2 (continuous line) and F8 (dashed line) *in situ* gelling systems, containing (A) 1 mg/mL and (B) 5 mg/mL in 0.1 N HCl. Bars represent mean±standard deviation (n = 3)

In order to investigate the ACN release mechanism, the release data was fitted to first-order kinetics, Higuchi law, and Peppas power law equations (Table 9.3). The regression coefficient (R^2) and the standard error of the regression (S) indicated that the Peppas power law equation had the best fit to the experimental data for all the formulations evaluated. The values for the release exponent (n) ranged between 0.25 and 0.31, indicating that the mechanism of ACN release from the gels was most likely controlled by Fickian diffusion, which occurs when these hydrogel systems are in their swollen state (Peppas et al., 2012). The release rate of compounds from these systems is controlled by diffusion through channels within the loose structure of the hydrogels (Tabata & Ikada, 1998). These results are in agreement with Rao and Shelar (2015) for *in situ* gelling solutions prepared with gellan gum.

Table 9.3 Release kinetics data fitted by first-order kinetics, Higuchi law, and power law

Form.	First-order		Higuchi		Peppas			
	R^2	S	R^2	S	R^2	S	k	n
1 mg/mL								
F2	0.6770	13.4002	0.8188	10.0381	0.9885	2.6784	0.45	0.25
F8	0.8197	11.2375	0.8568	10.0129	0.9909	2.6728	0.49	0.28
5 mg/mL								
F2	0.8423	10.7134	0.8655	9.8925	0.9917	2.6012	0.49	0.28
F8	0.8792	9.5575	0.9023	8.5958	0.9925	2.5212	0.47	0.31

Note: R^2 – regression coefficient; S – standard error of the regression; k – kinetic constant; n – release exponent

9.4.4 Potential Implications for the Administration of Anthocyanins

Talavéra et al. (2003) reported that the *in situ* gastric administration of 14 μmol/L of purified ACNs in Wistar rats for 30 min resulted in the average absorption of 25 % of the monoglycosides, depending on the compound structure. The concentration is in agreement with Crespy et al. (2002) and Felgines et al. (2007), and would result in

approximately 70 nmol of ACNs being administered to the animals directly in the stomach. The average absolute surface area of rat and human stomachs are $6.2x10^{-4}$ m^2 (glandular stomach) (Jarvis & Whitehead, 1980) and $5.3x10^{-2}$ m^2 (Snyder et al., 1975 cited by DeSesso & Jacobson, 2001), respectively. Normalizing the concentration investigated by Crespy et al. (2002) and Felgines et al. (2007) to the gastric surface area would result in approximately 2.9 mg to be administered in human subjects in 30 min, which indicates that the formulations prepared in this study with 5 mg of freeze-dried extract per mL could be used to investigate the absorption of ACN in humans with sustained release of these compounds over time.

It is worth noting that differences also exist between the gastric pH of humans (1–2) and rats (3–5), which could interfere with the ionization status of certain compounds such as ACNs. The reversible transformation of the main ACN structure in response to changes in pH is well characterized in the literature (He & Giusti, 2010). It is likely that at low pH observed in the human stomach, the predominant form of ACN will be the flavylium cation, whereas the carbinol pseudobase would be the main form at pH 3–6 in rat stomach (Brouillard & Dubois, 1977). It has been suggested that the uptake of ACNs by bilitranslocase would require the presence of at least one free negative charge, for example in the form of an anionic quinoidal base (Passamonti & Sottocasa, 1988; Passamonti, Vrhovsek, & Mattivi, 2002). It is possible that the higher pH found on the surface of the gastric mucosa (due to the presence of bicarbonate in the mucus layer or protons scavenged by mucins) (Li, Lieleg, Jang, Ribbeck, & Han, 2012) could lead to changes in the ACN structure, favoring the absorption; however, this needs further investigation.

Another important consideration is the subject's posture (Bennett, Hardy, & Wilson, 1984; Castell, Dalton, Becker, Sinclair, & Castell, 1992) and time of dosing relative to a meal (Washington et al., 1990). Comparing different postures, Bennett et al. (1984) showed that the gels emptied at faster and slower rates when the subjects lay on their left and right sides, respectively.

Washington et al. (1990) reported that an *in situ* gelling solution remained in the stomach for significantly longer periods of time when administered 30 min after a meal when compared to ingestion in a fasted state or immediately prior to a meal. The

consumption of the formulation in the fasted state resulted in a fast rate of emptying from the stomach, whereas the meal prevented the formation of the raft (Washington et al., 1990). Kahrilas et al. (2013) recently reviewed the etiology and implications of the so called “acid pocket”, which consists of an unbuffered area in the stomach that can accumulate acid for up to 90 min after a meal and would serve as a reservoir of gastric acid. Since the *in situ* gelling solution requires a low pH to form the gel and float, this “acid pocket” could be used as a platform to trigger the gelation process after a meal. For this reason, it is recommended that the ACN solution should be taken at least 30 min after a meal.

9.5 CONCLUSION

This study provides evidence that *in situ* gelling solutions could be used as a novel platform for the delivery of ACNs, based on *in vitro* results. Different formulations were evaluated for their gelling characteristics in simulated gastric pH, with further incorporation of ACN-rich freeze-dried extract prepared from haskap berries. The release profile indicated that the Peppas equation fitted the data better, suggesting that diffusion was most likely the mechanism of ACN release from the gels. These solutions are expected to remain in the stomach for longer periods of time than conventional systems and meals, which would contribute to modulate the release and absorption of ACNs with further implication for health-related properties. However, the appropriate amount of ACNs that should be released over time to result in significant benefits still has to be determined in humans.

Chapter 10 COMPARISON OF FLOATING MICROSPHERES AND *IN SITU* GELLING SYSTEM FOR THE DELIVERY OF ANTHOCYANINS

In the previous chapters, two novel floating delivery systems (microspheres and *in situ* gelling system) were developed as potential platforms for the purposes of increasing the retention time and modulating the release of ACNs from haskap berries in the stomach. In this section, the characteristics of these systems are first summarized and then compared to determine which is the more suitable platform for further study.

10.1 SUMMARY OF THE FLOATING DELIVERY SYSTEMS

The characteristics of the two floating delivery systems (FDS) developed and described in previous Chapters are summarized in Table 10.1 and a flowchart summarizing the processing steps required for their preparation is presented in Figure 10.1. It should be noted that freeze-dried haskap extract was used in both formulations and therefore its preparation was neglected from the comparison.

Based on the characteristics exhibited by the floating particles prepared in Chapter 8, microspheres prepared with 10 % acetic acid and a 1:4 $CaCO_3$ to alginate weight ratio were used as the most promising microsphere formulation for comparison with the *in situ* gelling system. In the case of the formulations investigated in Chapter 9, F2 and F8 containing 5 mg/mL of freeze-dried extract exhibited similar results. F8 was selected as the most promising *in situ* gelling system for comparison, as it contains more sodium bicarbonate and calcium carbonate than F2, which could be more effective for *in vivo* performance. The following section gives a detailed discussion comparing the two FDS platforms.

Table 10.1 Summary of properties for the floating delivery systems developed

Characteristic	Floating microspheres	*In situ* gelling formulation
Principle	Extrusion/gelation	*In situ* gelation
Buoyancy		
Floatation lag time	Immediately	Immediately
Floating capacity (%)	86.7	100.0
Chemical properties		
EE (%)	56.9	~100.0
ACN loading (%)	0.36	2.14
Release		
Mechanism	Diffusion	Diffusion
Peppas power law equation		
R^2	0.9723	0.9925
k	0.66	0.47

10.2 COMPARISON OF THE FLOATING DELIVERY SYSTEMS

The delivery platforms developed in this study are based on the ionotropic gelation of negatively charged alginate chains and calcium ions (Mørch et al., 2006). Alginates have been commonly used for the formation of films and encapsulation of bioactive components by the food industry due to their biocompatibility, low costs, and for being *generally recognized as safe* (GRAS) polymers (Nazzaro, Orlando, Fratianni, & Coppola, 2012). However, microspheres and *in situ* formulation differ by the manner in which the gelation occurs. In the former, the gel formation occurs in a gelation medium containing the divalent cations, leading to the formation of a monolythic system, i.e. the bioactive compounds of the haskap berry extract will be embedded in an alginate matrix.

In the latter case, the gel is formed in the stomach after the consumption of the alginate solution, forming a polymeric matrix.

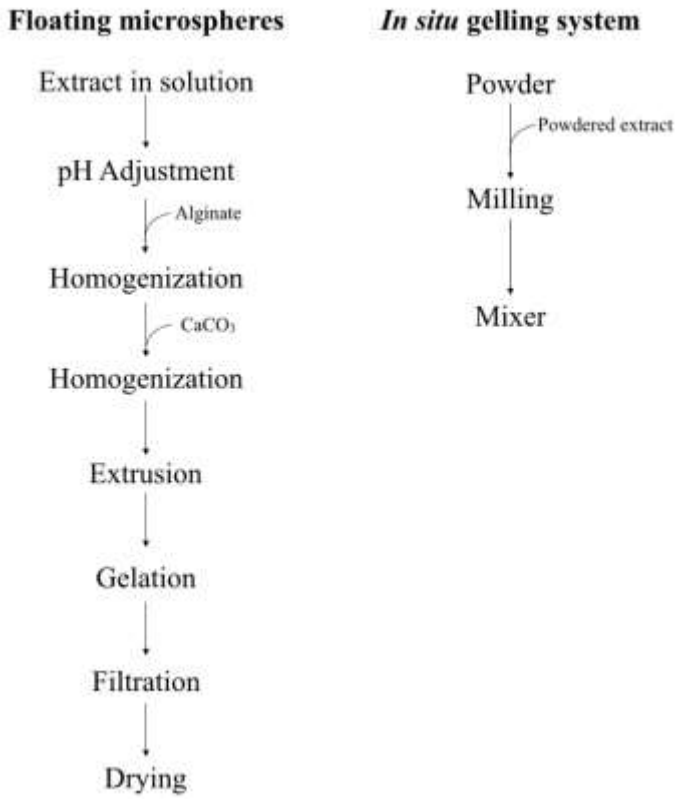

Figure 10.1 Flowchart summarizing the processing steps required for the preparation of floating microspheres (left) and *in situ* gelling system (right)

Both delivery systems incorporate one or more gas-generating compounds, namely calcium carbonate (in the floating microspheres) and both calcium carbonate and sodium bicarbonate (in the *in situ* gelling system). These compounds are necessary to reduce the density of the formulations and enable them to float above the simulated enzyme-free gastric fluid that was used in this study for *in vitro* characterization. The mechanism for gas generation is based on the following reaction of calcium carbonate in an acidic environment:

$$2H^+ + CaCO_3 \rightarrow Ca^{2+} + H_2O + CO_2$$

Different sources of the proton were provided in the formulations for the floating microspheres and *in situ* gelling system (acetic and hydrochloric acids) for the gelation/gas generation that occurred in the gelation medium and in the simulated "stomach". Acetic acid is a weak acid and, as such, is partly ionized in water. Chapter 8 has shown that a higher concentration of acetic acid was required to generate gas from the carbonate in the floating microparticles. Hydrochloric acid, on the other hand, is a strong acid and would contribute to the generation of gas almost immediately after the addition of carbonate. A potential issue with floating microspheres is that the CO_2 gas could permeate out through the porous alginate shell during drying, affecting the floating capacity of dry particles, as discussed in Chapter 8. Nonetheless, approximately 87 % of the dry particles floated immediately above the simulated gastric fluid. The *in situ* gelling formulation also floated instantaneously (within 2–3 s) to the surface of the fluid, and 100 % floatation was observed.

The *in situ* gelling formulation exhibited significantly higher *EE* and ACN loading capacity (measured as the amount of ACNs in a known amount of GRS) than the microspheres. The floating microspheres are interesting for tailoring the dose, as varying amounts of particles could be ingested if needed. However, because *EE* and loading are very low, larger amounts might be required to observe an effect *in vivo*, which could be unfeasible for administration and patient compliance. In the case of the *in situ* gelling system, once the required dosage is confirmed from *in vivo* studies, the amount of ACNs can also be tailored within the formulation as well as in adjusting the administered dose if necessary.

Both floating systems exhibited release profiles that indicated that diffusion was most likely the mechanism by which ACNs were released. A good fit of the experimental data was obtained with Peppas power law equation and differences were observed in the kinetic constant (k). A larger k, found for floating microspheres, indicates that the ACN release occurred faster than in the raft system. This difference can be seen when the release curves are plotted together (Figure 10.2). The raft system has a more sustained release of ACNs over time than the microspheres. The burst effect is more pronounced for the microspheres, with more than 60 % of the ACNs being released in the initial 30 min (compared to ~33 % from the raft), followed by little contribution to the overall

amount released and, ultimately, a plateau. Using the same analysis presented in Chapter 8 for measuring the agreement between curves, the difference (f_2) and similarity (f_1) factors confirmed that the release profiles are indeed different (f_2 = 41.54, with f_1 above 17 %).

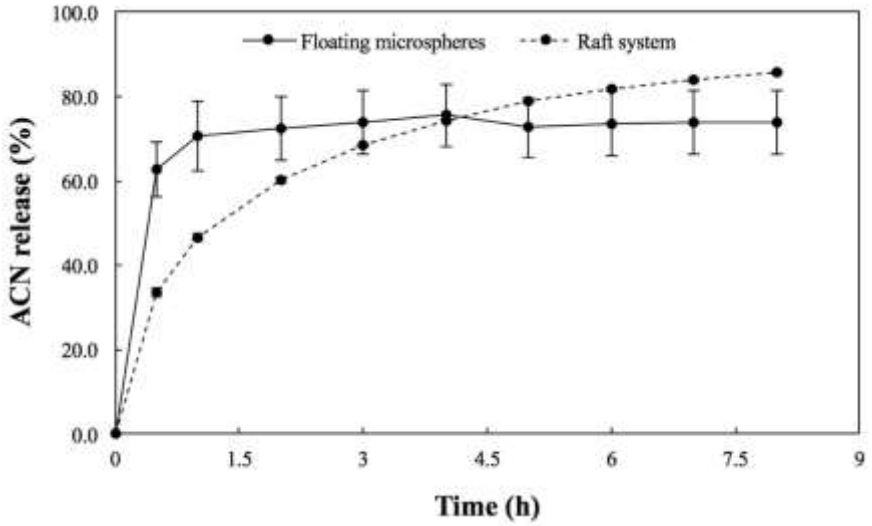

Figure 10.2 *In vitro* ACN release profile from floating microspheres (continuous line) and *in situ* gelling/raft system (dashed line) presented in Chapters 8 and 9, respectively. Bars represent mean±standard deviation (n = 3)

In relation to the processes required for the preparation of these delivery systems, the *in situ* gelling system offers several advantages over floating microspheres. Figure 10.1 shows that the process proposed for the preparation of the *in situ* gelling system is relatively simple as it does not require many steps. In addition, it does not require sophisticated equipment for its preparation. The floating microspheres, on the other hand, are prepared in various steps and would require a considerably large number of needles or nozzles for the extrusion step during production at a large scale. For the *in situ* gelling system, the scale-up is virtually unlimited.

For the reasons presented here, the *in situ* gelling system offers more advantages for the oral delivery of ACNs than floating microspheres and should be the subject of further research.

Chapter 11 CONCLUSION

11.1 SUMMARY AND CONCLUSIONS

This thesis presented the development and characterization of a floating (low-density) system for the delivery of ACNs extracted from haskap berries that could have greater retention in the stomach. Two strategies were investigated: microspheres containing a gas-generating compound (namely $CaCO_3$) and an intra-gastric floating *in situ* gelling system.

In **Chapter 2**, the current research on haskap berries was critically reviewed. Even though these fruits are fairly unknown in North America, they have a great potential to be used for the development of value-added products due to their high content of bioactive compounds. Different methods have been used to assess the bioactive content and biological relevance of haskap berries and other plant sources, and some limitations were identified (e.g. lack of specificity of the FC assay for phenolic compounds). Possible health benefits associated with the consumption of haskap berries were identified based mostly on *in vitro* and *in vivo* studies. Although haskap berries are commercially available in different food products, it was identified that further research is needed to determine the effects of processing on the bioactive content in order to preserve or maximize its potential as a functional food. In this context, encapsulation was suggested as a potential delivery system for bioactive compounds from these berries and to improve their stability.

In **Chapter 3**, the use of encapsulation techniques by the food industry was discussed for various bioactive components. Some limitations for a broader use of encapsulated products were identified, such as scale-up procedure, costs of new technology, balance between physicochemical characteristics of the encapsulate and sensory attributes, among others. In addition, it was indicated that the fate of the encapsulated compounds after consumption is often neglected. Recommendations for future work and regulatory aspects were also discussed.

Chapter 4 presented a review on the current knowledge of the PK of ACNs, which was used as a guideline to develop a theoretical PBPK model. This model is more

comprehensive than what had previously existed for ACNs as it takes into consideration some anatomic factors that have been overlooked. In addition, it provided further evidence for the involvement of the stomach in the absorption of ACNs. Further, in **Chapter 5**, this information was used as a basis for proposing gastroretentive systems as a strategy to increase the retention time of ACNs in sites where their stability and absorption are favoured. T2D was selected as a model for degenerative disease, as the consumption of ACNs has been shown to effectively manage and control it. However, a large bench-to-subject gap still exists and the use of GRS could aid in reducing the degradation of ACNs and modulating the delivery of compounds that have biological activity. Among the different GRS categories presented in this chapter, floating and raft-forming systems were identified as applicable to the food industry.

In **Chapter 6**, the UAE of ACNs from haskap berries was investigated. UAE is often referred to as an effective technique for the extraction of bioactive compounds that uses low temperatures and short time. The significant factors that impact the extraction were identified and optimized using PB and BB designs, respectively. For instance, it was shown that the solvent to solid ratio, solvent concentration, and time of extraction significantly affected the UAE. A highly positive and statistically significant relationship was found between TAC and TPC results. The model obtained by RSM was adequate to predict TAC (mean error of 1.28 %) and a good fit to the experimental data.

In **Chapter 7**, the extraction method was further modified to accommodate the larger quantities of extract required for further experiments. The extract was then encapsulated by ionotropic gelation into calcium-alginate particles, using BB design. In this study, the desirability function was used to optimize two responses simultaneously. These experiments were used as a basis for the development of floating microspheres, presented in **Chapter 8**. In order for the particles to float in a simulated gastric fluid, $CaCO_3$ was used as the gas-generating compound and two concentrations of acetic acid in the gelation medium were investigated. This acid reacted with the carbonate, releasing CO_2 gas. The incorporation of carbonate had an effect on the size, *EE*, and release of ACNs from the particles.

In **Chapter 9**, another strategy was proposed as a GRS and based on an intra-gastric floating *in situ* gelling formulation. This system contained sodium bicarbonate in

addition to the carbonate used previously. Thirteen base formulations were investigated to determine the suitable range of parameters, and the formulation was then further developed with the incorporation of an ACN-rich extract.

Chapter 10 summarizes and compares the characteristics of the two floating systems developed in this study. Both systems exhibited release profiles indicating that diffusion was the dominant mechanism of release. Based on the properties exhibited by floating microspheres and *in situ* gelling solution, the latter was selected as the more advantageous platform for the oral delivery of ACNs.

The **specific conclusions** relating to the original research objectives are as follows:

i) The optimal conditions for UAE of ACNs from haskap berries were: liquid to solid ratio 25:1 (mL/g), solvent composition of 80 % ethanol with addition of 0.5 % formic acid, ultrasound bath temperature of 35 °C for 20 min. Under these conditions, the TAC of 22.73 mg C3G/g DW was obtained experimentally.

ii) A novel system of floating microspheres with ACN extract was successfully developed. The microparticles were prepared by ionotropic gelation of alginate (9 %, w/w) with calcium ions ($CaCl_2$ at 2 %, w/v), using calcium carbonate as the gas-generating compound. Increasing the carbonate:alginate weight ratio from 0 to 3:4 resulted in different degrees of floatability, larger particles, higher encapsulation efficiency, and lower ACN release. Microspheres prepared with 10 % acetic acid and a 1:4 $CaCO_3$ to alginate ratio were the most promising microsphere formulation.

iii) A novel *in situ* gelling system with ACN-rich extract was successfully developed. The best formulation (F8) consisted of sodium alginate (3.5 %, w/v), sodium bicarbonate concentration (2 %, w/v), and calcium carbonate (2 %, w/v).

iv) Both the floating microparticles and *in situ* gelling systems exhibited release profiles that indicated that diffusion was most likely the mechanism of release.

v) The *in situ* gelling system is recommended for further development, due to a more favourable release profile and suitability for large scale industrial processing.

11.2 NOVEL CONTRIBUTIONS TO SCIENCE

This study provides insight into the delivery of ACNs that can potentially bridge the current bench-to-subject gap and is unique in many different aspects. For the first time, haskap berries were used as a source of ACNs for the development of the delivery systems. Current commercial products made with haskap berries are generally based on the use of whole fruits and are not targeted for a specialized application. In this study, the encapsulation of haskap berry extract is proposed as a potential technique to retain the activity of these compounds and prevent issues that are observed when using whole berries (e.g. perishability and seasonality) or extract (e.g. degradation during storage).

The PK of ACNs has been previously described by other authors; however, this is the first time that a PBPK model is described as a tool to predict their fate *in vivo*. By comparison, past reports have used a non- or one-compartmental analysis, which offers limited information of the PK. The proposed PBPK model is more comprehensive as it accounts for anatomic sites and factors that can have a contribution to the fate of ACNs after ingestion. As with any theoretical model, the one proposed in this thesis should be further verified and adjusted when appropriate experimental data is available.

Another unique contribution of this work is the identification of gastroretentive systems as platforms to modulate the release of ACNs where their stability and absorption are favoured. Two novel floating systems have been proposed, i.e. floating microspheres and an *in situ* gelling system. It was shown that they can modulate the release of ACNs in simulated conditions *in vitro*. The intra-gastric gelling system is unique as it does not require sophisticated equipment for its preparation and could be commercialized in a powder form for immediate dissolution prior to consumption, in a simple user-controlled preparation. In addition, a novel experimental protocol was developed to determine the release profile of the *in situ* gelling system. The release profile of pharmaceutical forms is often determined in USP apparatuses that can disrupt

the gel structure. The plunger system used in this study is simple, had little effect on the gel integrity and could be used in semi-dynamic assessments.

While many studies have reported the encapsulation of ACNs in different matrices, this study has explored a new delivery platform through the incorporation of gas-generating compounds which enables the system to float above simulated gastric content. The systems proposed here have the added benefit of utilizing alginate, a compound that has been commonly used by the food industry as a polymer for encapsulation, and other ingredients in the formulations that are approved for food applications. Most importantly, this thesis provides a comprehensive analysis of the potential of haskap berries in novel gastroretentive delivery systems for targeted health-promoting applications that can potentially bridge the current bench-to-subject gap.

11.3 RECOMMENDATIONS FOR FUTURE WORK

- The preparation of floating microspheres at a large scale should be investigated. The current system works well in a laboratory setting, however scaling up the process will require equipment that can accommodate larger throughput. The internal gelation technique (Poncelet et al., 1992, 1995) could be investigated as a potential method for the preparation of a larger number of particles. However, because the particle size can be reduced using this technique, the effect of processing parameters on particle properties should be investigated. Reduction of size may be beneficial for a broader application of the particles into various functional foods and nutraceutical products.
- The chemical stability of ACNs under adverse conditions and during storage was not evaluated in the present study. This is an important characteristic that should be evaluated so a prediction of shelf-life could be made. In addition, the physical stability of the systems (e.g. powder flowability and particle integrity under mechanical stress) should also be investigated as this could impact future applications of the product.
- The *in situ* gelling system is intended for oral consumption. The sensory attributes of this formulation should be evaluated and modified accordingly. For instance,

the taste might not be appealing for all consumers and this should be adjusted with the addition of flavours. Potentially, this system could be converted to a solid form (e.g. pill) that would mask unwanted flavours; however, any required modifications to the formulation would have to be further investigated.

- Independently of the system used, the platforms have to be evaluated in human volunteers to effectively confirm their retention in the stomach and, ultimately, if the time they remain buoyant is enough to improve the absorption and bioavailability. Based on these results, it might be necessary to adjust the dose accordingly.
- The incorporation of microspheres into the *in situ* gelling system should be investigated to determine the release profile of this combined system. The combination of both systems could reduce the initial burst in release and potentially lead to a zero-order kinetics profile.

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