SEQUENTIAL MICROWAVE EXTRACTION OF POLYSACCHARIDES AND PHENOLIC COMPOUNDS *FROM VACCINIUM MACROCARPON L.* AND THE EFFECTS OF IN-VITRO DIGESTION AND FERMENTATION

By

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ABSTRACT

Cranberry (*Vaccinium macrocarpon* L.) pomace was used to prepare an extract enrich with cell wall polysaccharides and phenolic compounds. Microwave-assisted extraction was investigated as a method to produce cranberry extract. Initial microwave-assisted extraction processes investigated the use of acidic, alkaline, and a sequential acidic-alkaline-based conditions at 36 W/g or 72 W/g cell wall material. Results demonstrated that the sequential acidic-alkaline extraction method was more efficient than acidic or alkaline extraction alone, and that the pomace was still rich in phenolic compounds and carbohydrates after acidic extraction. Sequential acidic-alkaline extraction provided 7.72-8.00% yield of extract, 51.06-52.22% total phenolic recovery, 4.20-5.27% carbohydrate recovery, with Gal A/Rha ratio of 17-25:1 after alkaline re-extraction. Both poly- and oligosaccharides were isolated upon the sequential microwave-assisted acidic/alkaline extraction. Hence, optimization using response surface methodology of the sequential acid-alkaline microwave assisted extraction method was conducted, due to the ability of further extract valuable cell wall components in a multi-step fashion with well-defined composition.

Four variables investigated during the optimization included microwave power (20-80 W/g cell wall material), pomace concentration (3.33-50.0 mg pomace/ml solvent), time (1-6 min), and sodium hydroxide concentration (0-1M NaOH). Predictive models were successful developed for the extract yield, and for the proportions and the recovery for each of carbohydrate and phenolic compounds. The optimal conditions were determined to be 65 W/g cell wall material, 4.73 minutes, 1.51 M sodium hydroxide, and 16.33 mg pomace/ml solvent, which generated 28.65% yield, 10.56% carbohydrate recovery, and 57.00% recovery of total phenolic compounds. The carbohydrate profile consisted of 38.10% uronic acids and 61.90% neutral monosaccharides. Gal A/Rha ratio was 15.8, indicating a high degree of branching of rhamnogalacturonan I. Size exclusion revealed a molecular weight distribution of the majority of polysaccharides in the 50-150 kDa range (45.64%). These optimal parameters were used during a scaled-up extraction to produce large amounts of extract for gastrointestinal digestion and fermentation. An enzymatically hydrolyzed extract was generated using Viscozyme L® to study the influence of smaller molecular weight polysaccharides during in-vitro fecal fermentation. Cranberry pomace (washed with 70% ethanol) and oligofructose were also investigated as controls.

The cranberry extract produced during the scaled up extraction process was subjected to TIM-1 simulated gastrointestinal digestion to assess the digestibility of the polysaccharides and phenolic compounds. TIM-1 digestion revealed that large proportions of glucose and galactose were found in the dialysates (29.07% and 28.57%, respectively), while uronic acids were found in the undigested effluents and chymes (16.30%). Phenolic compounds increased 2.3-fold during the digestion. During *in-vitro* fecal fermentation, the cranberry extract and the original cranberry pomace encouraged the growth of *Clostridia* cluster XIVa (Δlog bacteria = 1.3-2.5), while the enzymatically hydrolyzed cranberry extract and the oligofructose stimulated the growth of *Bifidobacterium* spp (Δlog = 0.5, 0.6, respectively). The highest concentrations of total short chain fatty acids were produced upon the fermentation of oligofructose (161.75 mmol/L) and the enzymatically hydrolyzed cranberry extract (159.67 mmol/L) followed by the original cranberry extract (158.60 mmol/L), and finally the cranberry pomace (145.49 mmol/L).

RÉSUMÉ

Le marc de canneberge (*Vaccinium macrocarpon* L.) a été utilisé pour préparer un extrait enrichi en fibres et en composés phénoliques provenant des parois cellulaires. L'extraction accélérée par micro-onde a été étudiée pour produire un extrait de canneberge. Les procédés d'extraction initiales ont été étudiés en utilisant des conditions acidique, alcalinisée et séquentielle acide-alcalin à 36 W/g ou 72 W/g de matériau de paroi cellulaire. Les résultats ont démontré que la méthode d'extraction séquentielle acide-alcalin était plus efficace que seulement l'extraction à l'acide ou à l'alcalin et que le marc était encore riche en composés phénoliques et des glucides après une extraction initiale par acide. Après un traitement à l'acide du marc, sa réextraction à l'alcalin a fourni 7.72-8.00% de rendement d'extrait, 51.06-52.22% de récupération des composés phénoliques, 4.20-5.27% de récupération des glucides provenant des fibres alimentaires, avec un rapport Gal A/Rha de 17-25:1. La méthode d'extraction acidique/alcaline a généré des poly- et des oligosaccharides. Considérant le potentiel du procédé d'extraction séquentielle acide-alcalin assisté par micro-ondes, son optimisation a été réalisé en utilisant la « response surface methodology » en vue de produire un extrait de canneberge enrichi en fibres et en composés phénoliques d'un grand intérêt.

Quatre variables ont été étudiées durant l'optimisation: puissance de micro-ondes (20-80 W/g de membrane cellulaire), concentration de marc (3,33-50,0 mg de marc/ml de solvant), temps (1-6 min) et concentration d'hydroxyde de sodium (NaOH 0-1M). Les conditions optimales ont été déterminées comme matériau de membrane cellulaire de 65 W/g, 4,73 minutes, hydroxyde de sodium 1,51 M et 16,33 mg de marc/ml de solvant, ce qui a donné un rendement de 28,65%, une récupération de glucides de 10,56% et une récupération de 57.00% des composés phénoliques totales. Des modèles prédictifs ont été développés pour le rendement d'extrait et pour les proportions et la récupération pour chacun des glucides et des composés phénoliques. Le profil des glucides se composait de 38,10% d'acide uronique et de 61,90% de monosaccharides neutres. Le rapport Gal A/Rha était de 15,8, indiquant un degré élevé de ramification du rhamnogalacturonane I. L'analyse de la masse moléculaire des glucides a révélé une distribution diversifiée dans laquelle des polysaccharides étaient de 50-150 kDa (45.64%). Les paramètres optimaux ont été utilisés lors d'une extraction à grande échelle pour produire de grandes quantités d'extrait de canneberge à utiliser dans l'étude de la digestion gastro-intestinale et de la fermentation

in-vitro. Un extrait hydrolysé enzymatiquement à l'aide de Viscozyme L® a été généré pour étudier l'influence des glucides de masse moléculaire plus faible au cours de la fermentation fécale *in-vitro*. Le marc de canneberge (lavés avec 70% d'éthanol) et l'oligofructose ont également été utilisés comme des contrôles lors de l'étude de la fermentation.

Une digestion gastro-intestinale simulée (TIM-1) de l'extrait de canneberge, enrichi en glucides et en composés phénoliques, a été conduit pour évaluer la digestibilité des glucides et des composés phénoliques. La digestion au TIM-1 a révélé que de grandes proportions de glucose et de galactose étaient présentes dans les dialysats (29,07% et 28,57% respectivement), tandis que les acides uroniques se trouvaient dans les effluents et les chymes non digérés (16,30%). Les composés phénoliques ont augmenté de 2.3 fois pendant la digestion. Pendant la fermentation fécale *in-vitro*, l'extrait de canneberge et le marc de canneberge ont favorisé la croissance de *Clostridia* cluster XIVa (changement en bactéries $\Delta\log = 1,3\text{-}2,5$), tandis que l'extrait hydrolysé enzymatique et l'oligofructose ont stimulé la croissance de la population de *Bifidobacterium* spp. ($\Delta\log = 0,5, 0,6$, respectivement). Les acides gras à chaîne courte totaux ont été produits, et les concentrations les plus élevées ont été obtenues après la fermentation d'oligofructose (161,75 mmol / L) et l'extrait hydrolysé enzymatiquement (159,67 mmol / L), suivi par de l'extrait original de canneberge (158,60 mmol / L), et enfin le marc de canneberge (145,49 mmol / L).

CONTRIBUTION OF AUTHORS

The present thesis consists of the three following chapters :

Chapter I involves a comprehensive literature review regarding the plant cell wall and its components as well as the common extraction methods for isolated plant cell wall materials. Also discussed is the human colonic microflora and the complex interactions between fermentation substrates and influence on bacterial growth and metabolic by-products.

Chapter II investigates the ability to isolate an extract rich in cranberry pomace cell wall components (oligo/polysaccharides; polyphenolic compounds) using microwave-assisted extractions under acidic, alkaline, and sequential acidic-alkaline conditions. The effects of extraction parameters were investigated and optimized using response surface methodology.

Chapter III examines the *in-vitro* digestibility of the cranberry oligo/polysaccharides /polyphenolic-enriched extract generated under optimal conditions from chapter II. Cranberry products, including the oligo/polysaccharides/polyphenolic-enriched extract, the enzymatically-hydrolyzed extract and the original cranberry pomace, as well as oligofructose were assessed for their *in-vitro* fermentability.

Connecting statements are included to provide a brief summary of the chapter and an introduction to the proceeding chapter.

Dr. Salwa Karboune, the MSc student's supervisor, provided a thorough guidance and supervision of the research work and a critical review of thesis materials before submission.

Erin Davis, the author, was responsible for execution of experiments and writing of the thesis.

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LIST OF ABBREVIATIONS

ANOVA:	Analysis of variance
Ara:	Arabinose
ddPCR:	Droplet digitalepolymerase chain reaction
g:	Grams
Gal A:	Galacturonic acid
Gal:	Galactose
GF4:	Fructo-furanosyl-nystose
Glu:	Glucose
HCl:	Hydrochloric Acid
HG:	Homogalacturonan
HPLC:	High performance liquid chromatography
hrs:	Hours
min:	Minutes
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
PCR:	Polymerase chain reaction
RG II:	Rhamnogalacturonan II
RGI:	Rhamnogalacturonan I
Rha:	Rhamnose
rpm:	Revolutions per minute
RSM:	Response surface methodology
s:	Seconds
SCFA:	Short chain fatty acid
SEC:	Size exclusion chromatography
TFA:	Trifluoroacetic acid
W:	Watts

INTRODUCTION

Cranberries (*Vaccinium macrocarpon)* are the berry fruits produced by shrubs in the upper Northern hemisphere. In fact, Canada is the second largest producer of cranberries in the world, with Quebec being an important producer in Canada (Watters, 2018). The increased production of cranberries and cranberry products will continuously generate more pomace, a potentially valuable material for the generation of added-value products. Cranberries are approximately 85-88% moisture, and depending on the efficiency of juice extraction techniques, there could remain up to 15% of the original weight of as pomace by-product (Česonienė & Daubaras, 2016). The pomace is largely insoluble plant cell wall material, rich in dietary fiber. Up to 60-70% of cranberry pomace is plant cell wall material, with minor amounts of protein, sugars, and fats. Cranberry pomace also remains rich in polyphenolic compounds, which are potentially valuable antioxidants if liberated from the plant cell wall (Holmes, Gilbert, & Rha, 1977; White, Howard, & Prior, 2010). The considerable amounts of cranberry pomace generated and the valuable plant cell wall components demonstrate that there are potentially other uses for cranberry pomace than animal feed or composting substrate.

The plant cell wall is rich in multiple types of polysaccharides, such as cellulose, hemicellulose, and pectin and its composition varies significantly depending on plant species (B. G. Smith, 2013). Many fruits are particularly rich in pectin, important for the ripening process (Talcott, 2007). Pectic polysaccharides are an incredibly diverse family of polysaccharides, consisting of homogalacturonan ([1,4]-α-D-galacturonic acid chain esterified with methyl esters), rhamnogalacuturonan I (repeating units of [1,4]-α-D-galacturonic acid and [1→2]-α-L-rhamnose with esterified neutral sugar side chains consisting of arabinose and galactose), and the very complex rhamnogalacturonan II ([1,4]-α-D-galactopyranosiduronic acid). Cellulose may also be present in more woody-type fruits ([1,4]-linked β-D-glucan) and hemicellulose ([1,4]-linked β-D-glycan heteropolymers) is often intertwined with pectic polysaccharides (Cosgrove, 2005; Voragen et al., 2009; Smith, 2013; Holck et al., 2014a). Lignin is another plant cell wall constituent associated with hemicellulose that is composed of phenolic compounds that are cross-linked to form an immense heteropolymer (Rabetafika, Bchir, Blecker, Paquot, & Wathelet, 2014; Yuan, Kapu, Beatson, Chang, & Martinez, 2016). While some studies have characterized the proximate composition and phenolic compounds in cranberry pomace, the chemical richness of its polysaccharide cell wall has yet to be exploited for better uses.

The abundance of dietary fiber contained within cell wall in cranberry pomace may have implications in the modulation of the diverse human colonic microflora. Non-starch polysaccharides are important fermentation substrates for bacterial such as *Bacteroidetes, Lactobacillus, Bifidobacterium, Enterococcus, Enterobacteriaceae, Clostridia*, and multiple other more species (Jandhyala et al., 2015). All inhabitant bacterial species utilise indigestible carbohydrate material differently (Louis, Hold, & Flint, 2014); however, these differences in metabolic capabilities are important for the modulation of bacterial populations and the symbiotic relationship between bacterial species (Van Laere, Hartemink, Bosveld, Schols, & Voragen, 2000; H.-D. Yoo, Kim, Paek, & Oh, 2012). The production of bacterial metabolites, namely short-chain fatty acids, is dependent not only on the type of bacteria and host, but also on the substrate used for the energy production; butyrate, acetate, propionate, and valerate are produced in very different proportions if fructooligosaccharides, inulin, cellulose, and various regions of pectin are fermented (Gulfi et al., 2007; Min *et al.*, 2015; Daguet *et al.*, 2016; Cantu-Jungles *et al.*, 2017a; Tabernero and Gómez de Cedrón, 2017). Therefore, the abundance of cranberry pomace presents a timely opportunity for the valorization of the remaining cell wall components, including cell wall polysaccharides and phenolic compounds. The richness in insoluble carbohydrate material is a key indicator that the polysaccharides present could have prebiotic properties that aid in modulating and contributing to a healthy gut microflora.

The overall objective of the present study was to develop a microwave-assisted extraction method for the isolation of an extract, rich in cell wall polysaccharides and phenolic compounds, from cranberry pomace and to study the *in-vitro* simulated human digestion and fermentation of the generated extract. This was achieved according to the specific objectives:

(1) To investigate the production of an extract enrich in the cell wall polysaccharides and the phenolic compounds from cranberry pomace using microwave-assisted extraction method.
(2) To optimize a two-step, sequential acidic-alkaline microwave-assisted extraction to produce a cranberry pomace extract rich in large molecular weight polysaccharides and phenolic compounds.
(3) To assess the digestibility of the extract rich in polysaccharides and phenolic compounds using simulated *in-vitro* digestion and evaluate its prebiotic activity using a continuous culture system inoculated with immobilized fecal microbiota.

CHAPTER I.

LITERATURE REVIEW

1.1 Introduction

Cranberries (*Vaccinium macrocarpon*) are the berry fruits produced by small shrubs in the upper Northern hemisphere. They are a close relative to blueberries, both members of the *Vaccinium* family. The most common destination for harvested cranberries is cranberry juice, which may be for direct consumption or to produce jellies and sauces. However, the process of juice extraction leaves behind a large amount of cranberry pomace; the leftover being mainly the cranberry cell wall with minor bioactive constituents such as polyphenols. Roughly 70% of cranberry pomace consists of soluble and insoluble fiber, while other minor constituents such as fat, protein, and carbohydrates make up the rest. However, the minor constituents are not present in sufficient quantities to merit their extraction (Holmes & Rha, 1978; White et al., 2010). As can be clearly seen, cranberry pomace presents a great opportunity for the extraction and study of dietary fiber – which may lead to the production of a carbohydrate by-product that is beneficial to the human gastro-intestinal tract.

1.2 Cranberry pomace

Around 95% of freshly harvested cranberries are destined for processing into several commercially available products such as juice, jams, and jellies. Commercial juicing is often the destination of cranberries, as their rich red colour and tart, astringent flavour is highly valued. However, the process of juice extraction leaves behind a large amount of cranberry pomace. Cranberries are approximately 85-88% moisture, and depending on the efficiency of juice extraction techniques, there could remain up to 15% of the original weight of as pomace by-product (Česonienė & Daubaras, 2016). There are relatively few studies and applications regarding the potential of cranberry pomace as an added-value product. Fruit Essentials Inc. in Wisconsin, USA, press cranberry seeds, left over from juicing, to obtain cranberry oil, which has its own health benefits. Other possible applications involve its use as a fertilizer or as animal feed, however these applications pose their own problems. Due to the large amounts of cell wall material remaining after juice production, there is potential for novel uses of cranberry pomace as a substrate for the generation of prebiotic carbohydrates. The Dieticians of Canada (2015) estimate that 50% of Canadians do not meet the daily dietary fiber intake recommendation, hence any advancement toward solving this problem could improve the gastrointestinal health of the general population.

Fresh cranberries contain approximately 12.5% total carbohydrates, minimal amounts of fat, protein, and minerals, as well as vitamin C and polyphenolic compounds (Table A1) (Kuzminski, 1996). After juice extraction cranberries contain approximately 62% dietary fiber as well as polyphenolic compounds (White et al., 2010). While pressing extracts many water soluble compounds, such as mono- and disaccharides, other less water soluble constituents and those that are bound to cell wall polysaccharides and proteins are left behind. Most polyphenols are water soluble, however some remain bound to the cell wall as cell wall bound phenolic compounds (Holmes & Rha, 1978). The pomace still retains a rich, pinkish-red colour, indicative that some phenolic compounds remain after juice extraction. An understanding of the plant cell wall is crucial for the extraction of cell wall material and bioactive compounds.

1.3 Plant cell wall components

1.3.1 General structure

Plants owe their structural integrity to the plant cell wall. The plant cell wall is what retains the living cells within the plant; plant cells differ from animal cells in that each cell is encompassed by a cell wall. Cellulose, hemicelluloses, and pectins are the main constituents of the plant cell wall, along with other complex polysaccharides such as xyloclucans, galactomannans, and heteroxylans. It is very important to note, however, that the cell wall constituents vary significantly between types of plants. Given this, monosaccharides rhamnose, fucose, arabinose, xylose, galactose, mannose, glucose, and acidic monosaccharides galacturonic acid, glucuronic acid, and 4-O-methylglucuronic acid are generally found as the main constituents of cell wall polysaccharides (B. G. Smith, 2013). Cellulose and hemicellulose bind together, creating the structural integrity of the cell wall. High molecular weight pectins form the middle lamella and are often regarded as the glue that holds all the cells together and in place (Cosgrove, 2005; Talcott, 2007).

Within the cell wall, many plant cells store starch as a form of glucose reserve. Starch does not impart a high osmotic pressure in the plant, whereas free glucose would impart a very high osmotic pressure. The aldehyde group on glucose is also readily oxidized to a carboxyl group, however, when glucose is stored in starch form, only the first glucose in the starch chain is available for oxidation. Amylose, amylopectin, and phytoglycogen are all found within the starch granule; amylose is the unbranched glucose polymer, amylopectin is a branched glucose polymer, and

phytoglycogen is a highly-branched form of starch. A combination of exoamylases, endoamylases, and phosphorylases are produced by the plant in order to access the glucose from the starch reserves to produce energy (Heldt & Piechulla, 2011). Starch falls under the "glucan" family, which simply indicates that it is a polymer of glucose monomers (Suzuki and Chatterton, 1993).

While many plants use starch as a glucose reserve, other plants use high molecular weight fructans, such as inulin, and low molecular weight oligosaccharides. Fructans are chains made from several fructose monomers (Suzuki and Chatterton, 1993). According to IUPAC (1982) the definition of an oligosaccharide is "a small molecule containing 2-10 monosaccharide residues connected by glycosidic linkages". Fructans, such as inulin, are store in some tubers such as parsnip and Jerusalem artichokes, as well as in onions (Heldt and Piechulla, 2011; Suzuki and Chatterton, 1993). Fructans are formed from a kestose starting molecule, where sucrose is linked with fructose molecules. 6-Kestose consists of a (6→2β)-linkage and 1-kestose consists of a (1→2β)-linkage. 6-kestose fructans are defined as levan type fructans, and the 1-kestose type as inulin type fructans. Neokestose type fructans have fructose chains linked to both glucose and fructose of the sucrose molecule; there is a (6→2β)-linkage between glucose and fructose, and a (1→2β)-linkage between fructose and fructose. Raffinose, stachyose, and verbascose are oligosaccharides containing a sucrose molecule with one or more galactose monomers bonded to it via glycosidic linkages. Oligosaccharides are found in many plants such as pulses and deciduous trees. Raffinoses are indigestible due to the absence of enzymes in the human digestive tract necessary for the hydrolysis α-galactosides, but are fermentable by certain species of bacteria found within the colon (Heldt & Piechulla, 2011).

1.3.2 Berry cell wall

Pectic polysaccharides make up a large amount of the plant cell wall of berries. All this pectin is responsible for the ripening process in many fruits. Enzymatic degradation reactions of the pectic substances in the middle lamella are mainly due to polygalacturonase and pectin methyl esterase. Hemicellulose and cellulose are often degraded by glucanases. As these carbohydrates are depolymerized, they are transformed into molecules with higher water solubility and contribute to the texture we associate with ripeness (Talcott, 2007).

1.3.3 Cellulose

The cellulose microfibril is made from unbranched molecules of (1,4)-linked β-D-glucan. To form the crystalline structure that provides incredible rigidity to the plant, the glucan residues of the cellulose chains crosslink with each other (Cosgrove, 2005; Fujino, Sone, Mitsuishi, & Itoh, 2000). Microfibrils are formed due to the interaction of cellulose chains through intermolecular and intramolecular hydrogen bonds, as well as van der Waals forces. Cellulose is relatively insoluble, however chemical modification to carboxymethylcellulose, methylcellulose and hydroxymethylcellulose has yielded more water soluble variants of cellulose that are used in the food industry and functional ingredients (B. G. Smith, 2013).

1.3.4 Hemicellulose

Hemicelluloses have a high binding affinity with cellulose and have a similar structure. (1,4)-linked β-D-glycans make up the backbone of the molecule with various sugars branching out from the backbone. The hemicelluloses that occur with greatest abundance in plants are xyloglucan and arabinoxylan, where xylose and arabinose branches, respectively, are linked to the glucose molecules of the backbone. There may also be other sugars such as fructose and galactose linked to the xylose branches (Cosgrove, 2005).

1.3.5 Pectic polysaccharides

Pectin is a large and diverse family of polysaccharides, consisting of a galacturonic acid backbone (α-1,4-D-galacturonic acid) with regions of rhamnogalacturonic acid and various neutral sugar side chains. Pectic polysaccharides may constitute up to 35% of the cell wall. Monosaccharides are often incorporated as component of side chains attached to the galacturonic acid backbone. Rhamnose is an important monosaccharide in that it forms the distinctive rhamnogalacturonan fractions. Homogalacturonic acid regions form the smooth region of pectin and hairy regions contain rhamnoglacturonic acid as well as other neutral sugars (B. G. Smith, 2013). Oligosaccharides derived from pectin are hence, very diverse (Babbar, Dejonghe, Gatti, Sforza, & Kathy, 2015; Holck, Hotchkiss, Meyer, Mikkelsen, & Rastall, 2014a). Rhamnogalacturonan I, rhamnogalacturonan II, homogalacturonan, and xylogalacturonan are all pectic structures with various chain lengths and residues. However, for a polysaccharide to be considered a pectin, the molecule must contain at least 65% galacturonic acid residues (Babbar et al., 2015; Endress & Mattes, 2012).A linear configuration of (1,4)-α-D-galacturonic acid residues forms

homogalacturonan, the most abundant pectic structure in the plant cell wall. The amount of methyl esterification at the C-6 position and acetylation at the O-2 or O-3 position can influence the ability of homogalacturonan to form gels. Cross-linkages with calcium cations at acetylated regions and also hydrophobic interactions amongst methyl groups between neighboring pectin chains are both important for gelling properties and depend on the degree of methylation and acetylation (Cosgrove, 2005; B. G. Smith, 2013; Voragen et al., 2009). Pectin methylesterase attacks the methyl groups at the C6 position, resulting in ripening of the fruit. Acetyl groups may also be acetylated to homogalacturonan (Holck et al., 2014a). The incorporation of xylose branches to homogalacturonan forms the pectin xylogalacturonan, where galacturonic acid residues may be methyl esterified (Cosgrove, 2005; Voragen et al., 2009). Xyloglucans are a glucan chain with xylose monomers linked at the C(O)6-position of the glucose residue in the glucan chain. Galactose and fructose monomers may also be linked to the xylose residues of xyloglucan, being linked to the the C2-position of xylose (B. G. Smith, 2013). Galacturonic acid and rhamnose make up the bulk of rhamnogalacturonan I. The backbone of rhamnogalacturonan one consists of repeating units of (1,4)-α-D-galacturonic acid and (1→2)-α-L-rhamnose. Neutral sugars galactose, and arabinose, and arabinogalactans may be esterified to the rhamnogalacturonan backbone at the C-4 position (Holck et al., 2014a; B. G. Smith, 2013; Voragen et al., 2009). These side chains are usually affected by endogenous enzymes and are cleaved off as the fruit ripens (B. G. Smith, 2013). Rhamnogalacturonan II is made up of several different types of sugars and is much more complex than rhamnogalacturonan I (Cosgrove, 2005). Arabinans and arabinogalactans, neutral pectic polysaccharides, are also found in conjunction with pectins (Cosgrove, 2005). A xylose backbone with α-L-arabinofuranosyl residue linked at the C3-position of the xylose monomer in the backbone and a α-D-glucuronosyl linked to the C2-position of the xylose monomers gives glucuronoarabinoxylans (B. G. Smith, 2013).

1.4 Pectin as a source of dietary fiber

Pectic oligosaccharides are derived from pectin and are not only useful for their functional properties as a food gelling agent, but also for their beneficial nutritional properties. Since they are indigestible by human enzymes, pectic oligosaccharides are considered a source of dietary fiber. Pectins are largely unaffected by the acidic conditions of the stomach, are not absorbed gastrointestinally, and are fermented by some microorganisms in the lower digestive tract to

ultimately encourage growth of beneficial colonic bacteria (Patel & Goyal, 2012). These oligosaccharides have also been shown to reduce the activity of several enzymes such as amylase, lipase, and trypsin and can even reduce serum cholesterol levels (Babbar et al., 2015; Endress & Mattes, 2012).

The prebiotic potential of pectic oligosaccharides is determined by a number of factors. The molecular weight distribution, type and amount of esterified sugars, as well as the degree of esterification all determine the properties of pectic oligosaccharides (Gómez, Gullón, et al., 2014). Low molecular weight pectic oligosaccharides have demonstrated greater prebiotic activity as compared to pectic oligosaccharides with higher molecular weight and also prevent pathogenic bacteria from attaching to epithelial cells in the colon. It has also been demonstrated that pectic oligosaccharides may offer some protection to colon cells in the presence of Shigella toxins and fatty acids may encourage apoptosis of adenocarcinoma cells in the colon (Babbar et al., 2015; Patel & Goyal, 2012).

1.4.1 Bacterial fermentation of pectin

Fermentation of pectin in the lower digestive tract takes place more toward the beginning of the colon. Short chain fatty acids, methane, carbon dioxide, and hydrogen gas are products produced by Bacteroides, Escherichia coli, Lactobacillus, and Bifidobacterium due to the fermentation of pectin. The growth Clostridium spp., Salmonella spp., and E. coli is generally decreased, possibly due to competitive growth of beneficial bacteria (Babbar et al., 2015; Patel & Goyal, 2012). Oligogalacturonic acid can also be produced due to incomplete fermentation. Important bacterial enzymes include polygalacturonase for breaking down the pectin backbone and 2-keto-3-deoxy-6-posphogluconate for fermentation. Pectin with a lower degree of esterification is more readily utilized by these colonic bacteria and tends to produce more short chain fatty acids (Endress & Mattes, 2012).

1.5 Polyphenols

Cranberries are rich in polyphenolic compounds, which not only confer their red colour, but also provide additional health benefits. Secondary metabolites that are produced by plants as a defense mechanisms, the basic polyphenol structure in plants is often a glycosylated (except for flavan-3-ols) 6-carbon ring with attached hydroxyl groups. Flavonoids, phenolic acids, stilbenes, and

lignans form the major families of polyphenols. Flavonoids consist of two benzene rings linked by a 3-carbon chain, where flavonols, flavones, flavanones, isoflavones, anthocyanidins, and flavanols are subclasses derived from this general structure. While many polyphenol profiles are specific to a specific plant material, flavanols and anthocyanins are the most abundant group of polyphenols. Water soluble anthocyanidins are the non-glycated form of anthocyanins and are responsible for many of the red, blue, and purple colours found in plants (Celep, Rastmanesh, & Marotta, 2014).

Studies have shown that polyphenols, such as those found in red wine and black tea, may prevent pathogen adhesion on the lining of the large intestine and also encourage growth of beneficial gut bacteria as an available carbon source. The response of microorganisms is highly dependent on the type of polyphenol. Some polyphenols exhibit a broad range inhibitory effect, whereas others demonstrate very little to no effect at all on bacteria. Meanwhile some beneficial bacteria show proliferation under the influence of polyphenols. Anthocyanins have been shown to encourage the growth of *Bifidobacterium* spp. and *Lactobacillus-Enterococcus* spp. The sugar fraction of the anthocyanin can be cleaved by lactic acid bacteria and used for malolactic fermentation (Celep et al., 2014; Duda-Chodak, Tarko, Satora, & Sroka, 2015).

Polyphenolic compounds are important in the prevention of cardiovascular disease as well as neurodegenerative disease (Celep et al., 2014). However, polyphenols have poor bioavailability in the human body, where only small molecular weight polyphenols are absorbed in the small intestine, being only 5-10% of the total dietary intake of polyphenols. De-glycosylation occurs before absorption. The unabsorbed, higher molecular weight polyphenols transit to the colon and are exposed to the bacteria residing there. Certain types of bacteria are capable of breaking down polyphenols via deglycosylation to produce aromatic acids, and breakdown of the carbon rings, which leads to the production metabolites that are absorbed or excreted by the human body. Bacteroidetes has much greater glycan degrading enzyme production than Firmicutes (Celep et al., 2014). After absorption, the metabolites travel via the portal vein to the liver, where further metabolism occurs. Certain bacterial species metabolize a given type of polyphenol, which leads to differences in microbial diversity given the types of polyphenols in the diet. Likewise, the consumption of foods rich in polyphenols help to modulate the types of bacteria present in the

large intestine, due to their prebiotic properties as a carbon source and via their inhibitory effect against pathogens (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013).

Cell wall differences in bacteria could affect how they respond to the presence of polyphenolic compounds. Metabolism of polyphenols can result in damage to bacterial cell walls due to the production of hydrogen peroxide, metal ion complexes, or by forming hydrogen bonds with the bacterial cell wall via hydroxyl groups. Whether bacteria are Gram negative or Gram positive can significantly affect the response to polyphenols. The production of communication molecules by bacteria, called quorum sensing, is important for cell growth and colonization. Polyphenols can interfere with quorum sensing for some bacterial species (Cardona et al., 2013).Dietary supplementation with flavonols has demonstrated an increase in Lactobacillus and Bifidobacterium species.

1.5.1 Effects of digestion and fermentation on phenolic compounds

Phenolic compounds are widely present in foods and are important antioxidants in the human diet, often carrying with them the fact of poor bioavailability in the human body. However, many undergo transformations upon digestion, improving their bioavailability. They have the potential for fermentation by bacteria in the colon as a substrate for the production of beneficial metabolites (Gil-Sánchez et al., 2017a).

Salivary digestion has the capacity to reduce total phenolic compounds significantly, potentially due the interaction between protein and fiber bound phenolics and their precipitation by saliva proteins (Campos-Vega et al., 2015). Gastric digestion has been shown to increase the amount of total phenolics and shows high recovery rates of polyphenol compounds and anthocyanins. The acidic milieu of the stomach can lead to the hydrolysis of larger phenolic compounds and also hydrolysis of bonds responsible for the association of phenolic compounds with the food matrix (Campos-Vega et al., 2015; Correa-Betanzo et al., 2014; Mosele, Macià, Romero, Motilva, & Rubió, 2015). Digestion occurring in the small intestine can also increase the amount of total phenolic compounds, where pancreatic protein interactions can occur. Flavanoids are affected by intestinal digestion, whereas tannins remain relatively stable (Campos-Vega et al., 2015). Anthocyanins are also reduced during intestinal digestion, due to the change from acidic to alkaline media, which can cause ring cleavage (Correa-Betanzo et al., 2014). However, the effects of

digestion depend greatly on the food matrix and composition, as demonstrated by Mosele et al. (2015). They discovered that total phenolic content decreased after intestinal digestion for both the pomegranate juice and pulp. However, it increased for the peel extract by almost 2-fold. The author theorizes that complex phenols within the food matrix are unextractable or are esterified to cell wall constituents, making them undetectable or unextractable when determining phenolic content present in the original, indigested material.

The structure of phenolic compounds significantly influences their absorptivity, and their association with cell wall polysaccharides influences their transport to the colon as a fermentation substrate (Campos-Vega et al., 2015). Monomers and some dimers are readily absorbed through the small intestine, but larger phenolic compounds must be metabolized by the gut microbiota. Digestion and fermentation showed that benzoic acids, phenols, phenylacetic acids, phenylpropionic acids, valerolactones, valeric acids, and cinnamic acids are the most bio-accessible metabolites generated from grape pomace (Gil-Sánchez et al., 2017a). Fermentation of polyphenols in the colon leads to the generation of metabolites that are absorbed by the epithelial cells in the colon, increasing bioavailability. Structural changes include alkaline cleavage of the benzopyran ring and the formation of phenolic compounds (Correa-Betanzo et al., 2014). Species within the *Bacteroides, Enterococcus, Eubacterium, Clostridium, Bifidobacterium, Lactobacillus, Butyrivibrio*, and *Peptostreptococcus* families are responsible for the breakdown of polyphenols (Jandhyala et al., 2015).

1.6 Extraction of cell wall components

1.6.1 Chemical extraction

1.6.1.2 Acid extraction

Commercial pectins are often extracted using acids at varying concentrations and high temperature conditions to generate pectin with high gelling capacity. Mineral acids generally provide high yield and affordability. Hydrochloric acid is often a popular choice for extraction (Jafari, Khodaiyan, Kiani, & Hosseini, 2017; Raji, Khodaiyan, Rezaei, Kiani, & Hosseini, 2017; Seggiani, Puccini, Pierini, Giovando, & Forneris, 2009; Yuliarti, Goh, Matia-Merino, Mawson, & Brennan, 2015). The type of acid used for extraction can have a significant impact on the yield and quality of pectin generated. Lemon peel extracted at varying concentrations of HCl, HNO_3, and $H2SO_4$ generated higher yields of pectin with increasing concentration. However, $H2SO_4$ generated the poorest

yield, which the authors attribute to the penetrability of the plant cell wall by different acids. Increasing concentration of all acids decreased the degree of methylation due to the hydrolysis of ester bonds (Seggiani et al., 2009). Jafari et al. (2017) also noted that extensive heating and extraction time during acid hydrolysis on carrot pomace can cause the hydrolysis of ester bonds. It was also suggested that higher pH values cause the aggregation of pectin in the plant material, making it difficult to extract.

Investigation into the use of organic acids instead of mineral acids has become popular due to a more environmentally friendly focus. Citric acid used to extract pectin from kiwi fruit pomace resulted in pectin with a lower arabinose concentration compared to enzyme extracted pectin, suggesting that acid causes the breakdown of arabinofuranosyl linkages in arabinan chains. The presence of xylose, mannose, and glucose after extraction indicate the breakdown of hemicellulose and cellulose. It was also noted that the acid extracted pectin was highly linear, indicating that acid extraction causes hydrolysis of branched regions (Yuliarti et al., 2015).

1.6.1.3 Alkaline extraction

Alkaline extraction is less widely used for extraction of plant cell wall polysaccharides and is often applied in the extraction of hemicellulose and for alkaline soluble or ester-bound pectins (Saulnier & Thibault, 1987; Yuan et al., 2016). Extraction using an alkaline medium will also cause hydrolysis of homogalacturonan chains due to β-elimination as well as de-esterification (Khodaei & Karboune, 2013; Müller-Maatsch et al., 2016). Extraction of cell wall polysaccharides under alkaline conditions causes the saponification of the ligin-hemicellulose ester bonds, leading to a co-extraction of hemicellulose and lignin. Cellulose degradation is also possible and is attributed to a “peeling phenomenon” which ultimately results in a high content of glucose within the monosaccharide profile of the extracted cell wall material (Rabetafika et al., 2014; Yuan et al., 2016).

1.6.1.4 Aqueous extraction

Extraction of plant cell wall polysaccharides has been employed largely using heated or pressurized and heated water along with varying time and temperature, largely with the goal of creating an environmentally friendly extraction process. Water extraction has been used as a first extraction step in the characterization of pectin, often yielding both neutral and uronic sugars

(Concha Olmos & Zúñiga Hansen, 2012; Cserjési, Bélafi-Bakó, Csanádi, Beszédes, & Hodúr, 2011). However, water soluble fractions of plant cell wall material do not always imply that these fractions contain pectic polysaccharides. For example, cactus cladode flour extracted with water at 80 °C for 20 min contained mostly mucilage gum (Pérez-Martínez et al., 2013). Another problem with hot aqueous extraction is lower yields of pectin, often due to the extraction of other cell wall polysaccharides. More recent techniques include the application of ultrasound an microwaves because of their penetration into cell wall material and greater yields of pectin and other polysaccharides (Huaguo Chen, Zhou, & Zhang, 2014; Nobre, Cerqueira, Rodrigues, Vicente, & Teixeira, 2015; Zhu et al., 2014).

Pressurized hot water, or referred to as "hydrothermal", extraction has been investigated for its capability of extracting pectin and hemicellulose, as well as generating oligosaccharides. Under conditions of elevated temperature and pressure, polysaccharide hydrolysis will occur due to the availability of hydronium ions created by autoionization of water as well as from the presence of organic acids within the plant material. Unfortunately, the reaction lacks specificity and can have other extraction effects on the plant material (Kurdi & Hansawasdi, 2015; Vegas, Alonso, Domínguez, & Parajó, 2004).

Cassava pulp, rice bran, and spent brewer's grain have been successfully treated hydrothermally to yield maximum amounts of potentially prebiotic xylooligosaccharides and low amounts of monosaccharides (Gómez, Míguez, Veiga, Parajó, & Alonso, 2015). Hydrothermal treatment also preserved the degree of methylation and acetylation of pectic oligosaccharides obtained from lemon peel wastes, orange peel wastes, and sugar beet pulp, however, hydrothermal treatment resulted in the loss of arabinose and galactose, owing to the degradation of arabinan and arabinogalactan constituents of pectin (Gómez, Gullón, et al., 2014; Gómez, Gullón, Yáñez, Schols, & Alonso, 2016). Apple pomace extracted using hot, pressurized water in an autoclave resulted in pectin with lower molecular weights and compared to commercially available pectins, where increased time and temperature caused breakdown of homogalacturonan. Neutral monosaccharides were significantly affected by this treatment, where the arabinose content was notably lower than previous studies, most likely due to the degradation of hairy regions. Hemicellulose was also extracted due to the large amounts of glucose present (X. Wang & Lü, 2014).

1.6.2 Enzymatic extraction

1.6.2.1 Cell wall degrading carbohydrases

There exists several classes of enzymes that can be applied to the degradation of specific cell wall components. Individual enzymes or multi-enzymatic preparations can be used for a variety of applications, ranging from the isolation of a specific cell wall polysaccharide to the hydrolysis of polysaccharides into oligomers and monomers.

Enzymes are highly specific, catalytic proteins that accelerate chemical and biological reactions by lowering the activation energy of the reaction with the help of an active site that can bind specifically with a substrate, making it readily accessible to a reactant (Holck, Hotchkiss, Meyer, Mikkelsen, & Rastall, 2014b; Powar & Chatwal, 2007). In response, enzymes are considered environmentally friendly because they allow reactions to be executed without the need of harsh reactants and extreme energy requirements, such as heat. They are also reusable to a certain extent, if appropriately maintained on a support material (Holck et al., 2014a).

1.6.2.2 Non-pectin degrading enzymes

The plant cell wall is composed of a pectin matrix that entwines cellulose microfibrils, proteins, hemicellulose, and lignin. Plant cell wall degradation requires a multitude of different enzymes that are generally produced by fungi (Grassin & Coutel, 2009). Glycosidic bonds are cleaved by glycoside hydrolases and they may have exo- or endo- activity, or both for a synergistic effect. Cellulases, which are β-1,4-glucanases, degrade cellulose to cellodextrin, which is then degraded by β-glucosidase to glucose. Xyloglucans, xylans, and galactomannans are non-cellulosic cell wall polysaccharides that are often referred to collectively as hemicellulose. Xyloglucan is degraded by the same enzyme as cellulose, endo-β-1,4-glucanase, since the backbone is β-1,4-linked glucose. endo-β-1,4-xylanases hydrolyse chains of xylan and mannans are hydrolyzed by 1,4-β-D-mannan mannohydrolase and 1,4-β-D-mannopyranoside hydrolase (Kubicek, Starr, & Glass, 2014).

1.6.2.3 Pectin Degrading Enzymes

The use of enzymes that are specific to certain bonds in the pectic structure can lead to hydrolysis that provides specifically structured and isolated poly- and oligosaccharides, which may be important for elucidating bioactivity, or can degrade these larger sugars down to monomers (Holck et al., 2014a).

Polygalacturonases are responsible for glycosidic bond hydrolysis of the uronic acid backbone, whereas pectin and pectate lyases cause the breakdown of the uronic acid backbone by a non-hydrolytic process called β-elimination. If there are regions of low esterification, endo-polygalacturonase will hydrolyze the α-(1,4) linkage of the galacturonic acid backbone. The terminal end of the galacturonic acid chain will be hydrolyzed by exo-polygalacturonase. Highly methylated backbones are generally hydrophobic, referred to as pectin, whereas the demethylated form is extensively charged and is referred to as pectate. Pectin and pectate lyase breakdown these chains using β-elimination, generating free reducing ends and an unsaturated uronic acid residue. Pectate lysase is calcium ion dependent (Babbar et al., 2015; Kubicek et al., 2014).

Pectinmethylesterase is capable of hydrolyzing methyl esters esterified to the galacturonic acid chain to release methanol. Pectin acetyl esterase acts in a similar fashion, except that it cleaves acetyl groups from the backbone. Rhamnogalacturonic residues are often hydrolyzed by rhamnogalacturonan hydrolase and rhamnogalacturonanlyase. Arabinofurosidase, endoarabinase, endogalacturonase, and exoarabinase release side chains from the rhamnogalacturonan chains. Exo-active enzymes, such as rhamnogalacturonan acetyl esterase and β-galactosidase are capable of hydrolyzing their respective residues at the terminal ends of the side chains and of the pectin backbone (Babbar et al., 2015; Grassin & Coutel, 2009).

1.6.3 Microwave assisted extraction

An array of novel extraction techniques have been developed with the goal of "green" extraction methods using ultrasonics, high pressure, and supercritical fluid and microwave systems. Green extraction refers to techniques that involve the use of reduced solvent volumes, non-toxic and non-pollutant solvents, as well as reduced extraction times. Microwave extraction is superior to conventional and novel methods of extraction. Microwave extraction fulfills many of the aforementioned "green" extraction requirements, is energy efficient and space efficient. Some limitations involve a post-extraction filtration or centrifugation step, poor extractability of non-polar compounds, solvent limitations, and the generation of high temperature that degrade thermolabile molecules.

Microwave assisted extraction of bioactive plant compounds has been studied extensively across a wide array of plant materials. Electromagnetic waves alter the plant cell wall structure, leading to accelerated extraction and higher yields of desired plant compounds. Contrary to conventional extraction, the energy generated from electromagnetic radiation is dispersed volumetrically in the solvent resulting in a mass and energy gradient that flows in the same direction. In conventional extraction, the energy and mass transfer occur in opposing directions. Dipole rotation and ionic conduction drive energy transfer and heating of the solvent. Heating due to dipole rotation occurs due to rotation and movement of the dipoles of a molecule in the presence of an electromagnetic field, whereas ionic conduction heats the solvent as a result of the electrophoretic movement of ions, such as salts, in the presence of an electromagnetic field. Molecules will shift to align with the electromagnetic field. The friction generated from the movement of these particles generates heat within the solvent and the material (Veggi, Martinez, & Meireles, 2013).

The choice of extraction solvent is ever important for extraction efficiency, given that solvents without a dipole or ions will not absorb microwave energy and will not generate heat. Often, the solvents used in conventional extraction cannot be used for microwave assisted extraction for this reason. Solvents can be mixed to increase polarity; the more polar solvents will absorb electromagnetic radiation and generate heat, transferring energy to the non-polar solvents that do not absorb microwaves, however, the change in polarity can also have an effect on what type of compounds are extracted due to their polarity. Salts can also be added. The solvent must also interact with the sample and the targeted extract compound for removal from the sample matrix. Superheating can occur, especially in systems and samples containing water, where microwave absorption is higher than dispersion. Sample matrix viscosity also has an effect on microwave absorption, since samples that are viscous will inhibit dipole rotation and ionic movement (Veggi et al., 2013).

Several factors influence the ability of electromagnetic radiation to generate energy within a solvent or material. The degree to which a material can interact with electromagnetic energy is called permittivity (ϵ') or the dielectric constant which determines how much of the electromagnetic energy is reflected at the air-sample interface and how much energy actually enters the material; the loss factor is the real part. The loss factor (ϵ'') is the imaginary part and

determines the efficiency of the transformation of the absorbed energy into heat, or how effectively the material can diffuse energy to neighboring molecules (Veggi et al., 2013).

The extraction process occurs in a series of steps; the relationship between extraction time and extract recovery is not linear. The solvent must first enter the solid material, after which cell wall degradation and solubilization can occur. The desired compound to be extracted is then carried out of inside of the cell wall material to the surface and then into the extraction solvent, where the extract is completely separated from the cell wall material (Veggi et al., 2013). Microwave assisted extraction can occur in the presence or absence of solvents. Solute solubility should not be regarded as a limiting factor to extraction, since the solvent to solute ratio is usually high and the extracted compounds are generally solubilized in the solvent. The rate of dissolution of the extract into the solvent is governed by Fick's law. Diffusion occurs from an area of high concentration (the solid matrix) to low concentration (extracted compound in a relatively larger volume of solvent). Solvent-free microwave extraction involves the movement of free and bound water within a solid matrix via capillary pores, which ultimately generates heat (Veggi et al., 2013). There are other extraction parameters that need to be considered for optimal extraction. The ratio of solid to solvent is also important, and most studies report between 10:1 to 20:1 solvent-solid ratio as being optimal. Large volumes require more energy and time to heat to the optimal extraction temperature. Stirring aids in mass transfer via accelerated desorption of the target compounds from the sample matrix. Extraction time is also an important parameter; prolonged extraction time can cause overheating of the solvent, causing damage to thermolabile compounds. Extraction cycles can be used to prevent degradation of sensitive compounds, where new solvent is added after each extraction in order to fully extract the target compound(s) from the matrix. Extraction temperature is also influenced by microwave power; greater microwave power increases the amount of energy and hence heat generated. There is a direct relationship between the amount of sample, power, and time. Determining optimal conditions that balance good yield and degradation of thermolabile components is crucial. Other parameters, such as surface area can affect extraction efficiency. Greater surface area enhances extraction efficiency and smaller particle size are more easily penetrated by microwaves. Particle sizes anywhere between 100 μm to 2 mm are typically used and the sample matrix can be pre-soaked in solvent to improve extraction yield. Bound water

within the cell matrix can also improve extraction efficiency due to heating and evaporation, which results in internal pressure and eventually cell rupture (Veggi et al., 2013).

1.7 Applications of green extraction methods

1.7.1 Applications of cell wall carbohydrases

The use of plant cell wall degrading enzymes are becoming widely appreciated for their numerous applications as pre-treatments to extraction of bioactive components of plant materials. A multi-enzymatic preparation has been used to improve protein extraction from green tea residues due to extraction interference with non-protein components such as polyphenols and carbohydrates. The pre-treatment resulted in increased protein recovery and quality, and less consumption of alkali compared to the traditional method (C. Zhang, van Krimpen, Sanders, & Bruins, 2016).

Volatile oil extraction has also been improved with the use of enzymatic pre-treatment. Plant cell wall degrading enzyme mixtures were used as a pre-treatment to steam distillation for volatile oils from fennel seeds. Oil yields were increased compared to the traditional method and varied depending on the capability of the enzyme to degrade seed carbohydrates. All enzymes resulted in a reduced need for solvent and energy consumption (Baby & Ranganathan, 2016).

Phenolic compounds in plant materials are often bound to the plant cell wall, making extraction difficult. Plant cell wall degrading enzymes have been used to investigate their effect on the polyphenolic profile of lentils as well as their antioxidant activities. All lentil flours treated with enzymes and enzymatic preparations showed an increase in antioxidant activity compared to the untreated lentil flour. This is most likely due to the release of cell wall bound phenolic compounds by the action of the enzymes (Duenas, Hernandez, & Estrella, 2006). Wine making by-products have also been subjected to the use of cell wall degrading enzymes for the release of insoluble, bound phenolic compounds. It was found that carbohydrate hydrolysis as a result of enzymatic treatment released more insoluble bound phenolic compounds (De Camargo, Regitano-D'Arce, Biasoto, & Shahidi, 2016). Unripe, homogenized apples have also been subjected to enzymatic treatment for the release of bound phenolic compounds. It was shown that enzymatic treatment with cell wall carbohydrases increased the total phenolic content approximately four times more than that of the control (Zheng, Hwang, & Chung, 2009).

Plant carbohydrases are also useful for aiding industrial food processors. Industrially, many pectinases are used in order to increase juice yields by decreasing viscosity by breaking down the plant cell wall (Grassin & Coutel, 2009). Cactus cladodes are rich in viscous mucilage which hinders juice processing. Treating the crushed cladodes resulted in a 3-fold decrease in viscosity and an increase in polyphenolic contents (Kim et al., 2013). The juicing industry, as well as other fruit and vegetable processors, product large amounts of potentially valuable food waste. Onion skins have been treated with different cell wall degrading enzymes to produce potentially prebiotic oligosaccharides. It was determined that different enzymes can produce different mixtures of oligo and monosaccharides depending on their exo- or endo- activity as well as incubation time M2 (Babbar et al., 2016).

Other applications aim to create greener products of non-food value. A multi-enzymatic cell wall degrading preparation has been used to investigate the production of a green, bio-based adhesive from defatted soy flour. Adhesive properties are a result of protein-sugar cross-linkages which requires reducing sugars. Reducing sugar content was increased up to 8% by pre-treating the flour with the enzyme mixture (N. Chen, Zeng, Lin, & Rao, 2015). A similar result was deemed desirable in another study involving the pre-treatment of sugarcane and agave bagasse for the production of biofuel. Desirably high amounts of reducing sugars were obtained (31-53% increase) when bagasse was pretreated with enzymes compared to steam and alkali treatment controls (Hernandez-Salas et al., 2009). Clearly, plant cell wall degrading enzyme have enormous potential for the production of many valuable products deemed for human consumption and use.

1.7.2 Microwave assisted extraction of polysaccharides

Microwave extraction of plant cell wall polysaccharides has been widely applied to both fruit and vegetable waste streams as a method of breaking down cell wall material. It is a rapid method and often requires lower solvent volume compared to traditional methods of extraction, and often provides better yields (Bélafi-Bakó, Cserjési, Beszédes, Csanádi, & Hodúr, 2012; Liew, Ngoh, Yusoff, & Teoh, 2016; Seixas et al., 2014). A high solvent to raw material ratio dissipates microwave energy to plant tissues, leading to pressure build-up within the cell wall from the production of water vapour. This alters the physical properties via cell rupture and enhances the contact between the extraction solvent and the polysaccharide containing material, leaching of cell contents, such as polysaccharides (Dong, Lin, et al., 2016; C. S. Kumar, Sivakumar, & Ruckmani,

2016; Liew et al., 2016; Seixas et al., 2014). Higher microwave power infers higher solvent temperature, leading to tissue softening and an increased mass transfer rate and dissolution of polysaccharides from the plant matrix into the solvent (Dong, Zhang, et al., 2016; Lefsih et al., 2017). It is imperative to recognize that excess heating and length of exposure can eventually cause thermal decomposition of polysaccharides, leading to decreased yields and smaller molecular weight polysaccharides (Prakash Maran et al., 2014; Dong et al., 2016b).

1.7.3 Phenolic compound extraction using alkaline media and microwave energy

Lignocellulosic biomass from plant waste streams is a potentially rich source of phenolic compounds. Lignin belongs to the secondary cell wall of plants, where ether and ester bonds link phenolic compounds to lignin and polysaccharides. Phenolic compounds include ferulic acid, vanillic acid, syringic acid, p-coumaric acid, gallic acid, vanillin, and p-hydroxybenzoic acid (Akpinar & Usal, 2015). The bonds forming the complex are broken down under alkaline (NaOH 1-4M), high temperature, or high pressure conditions (Buranov & Mazza, 2009; Nenadis, Kyriakoudi, & Tsimidou, 2013). Microwave extraction has been used to extract phenolic compounds with high antioxidant activity and yield with the benefits of quicker extraction and reduced solvent and energy consumption. Microwave extraction can be used with traditional extraction solvents such as ethanol and methanol, however water, NaOH and HCl have also been investigated (Bouras et al., 2015; Valdes, Vidal, Beltran, Canals, & Garrigos, 2015). Extensive exposure to microwave energy can cause degradation of phenolic compounds, while the extraction of hemicellulose and some cellulose is also observed under harsh conditions (Carvalho, Sevastyanova, Queiroz, & Colodette, 2016).

Table 1. Summary of advantages and disadvantages of selected extraction techniques.

Method	Advantages	Disadvantages
Conventional acid extraction	• Affordable • Isolation of high gelling pectins • High pectin yield	• Less environmentally friendly • Hazardous concentrated acids • Hydrolysis of rhamnogalacturonan
Conventional alkaline extraction	• Affordable • Isolation of ester-bound pectic polysaccharides • Isolation of hemicellulose, lignin, and phenolic compounds	• Less environmentally friendly • Hazardous concentrated bases • Hydrolysis of homogalacturonan
Hydrothermal aqueous extraction	• Affordable • Environmentally friendly • Limited hydrolysis on plant polysaccharides	• Often requires use of high pressure and temperature • Lack of specificity • Potential to cause extensive degradation to plant cell wall polysaccharides due to high heat and pressure.
Enzymatic extraction	• Environmentally friendly • High specificity • Reusable	• Expensive • Time consuming
Microwave assisted extraction	• Environmentally friendly • Energy efficient • Fast • Applicable to acid, base, and aqueous media	• Specialized equipment needed • Efficiency highly dependent on solvent • Non-specific • Thermal degradation of heat labile compounds

As discussed in the preceding sections, plant cell wall material derived from industrial processing is a rich source of polysaccharides and phenolic compounds. The isolation of these plant cell wall constituents can be performed using numerous techniques to generate polysaccharides and phenolic compounds with varying characteristics that may be of interest from a health stand point. The following sections will demonstrate the importance of using these types of by-product cell wall components as substrates that are fermentable by microorganisms living in the human gastro-intestinal tract.

1.8 The Human digestive tract

The digestive system is responsible for extracting all of the important nutrients from foods we eat. These micro and macro nutrients are important for functioning during out day-to-day lives. The various systems within the gastrointestinal tract (GIT) work together to supply the human body with what is needed. Digestion, absorption, secretion, excretion, and motility are all functions that occur within the gastrointestinal tract (M. E. Smith & Morton, 2011). The digestive system is comprised of an upper and lower digestive system, both having their major functions. There are four layers lining the digestive tract. The external layer is a network of connective tissue known as the serosa, important for holding organs in place within the digestive system. The muscularis externa lines the inside of the serosa and compromises longitudinal and circular muscles. The nervous system is responsible for controlling contractions of the longitudinal and circular muscles, the former being responsible for the length of the digestive system and the latter for the diameter. Nerves, blood and lymphatic vessels are connected via the submucosa, which lines the inside of the muscularis externa. Epithelial cells are found within the mucosa, the layer with the most intimate contact with the inside of the digestive system. They exist as folds which allow them to have a high surface area, important for absorption and secretion. The folds present in the mucosa layer of the stomach are important for expansion, whereas in the small intestine the folds are necessary for nutrient uptake. Epithelial cells are important for the efficient functioning of the digestive system; they secrete mucus, gastric fluids, and even hormones (McDowell, 2010).

1.8.1 The oral cavity, esophagus, and stomach: "upper" GIT

The mouth is the beginning of the upper digestive tract, where food enters and is mechanically broken down by chewing. This increases the surface are of the food, necessary for efficient breakdown via digestive enzymes. Salivary amylase is also secreted (McDowell, 2010).

Once swallowed, the bolus reaches the stomach, and it is churned and mixed with gastric fluids to produce chyme. Differentiated cells within the mucosa layer secrete the inactive enzyme pepsinogen and hydrochloric acid. Hydrochloric acid not only unravels proteins within the bolus, but it also activates pepsinogen and prevents pathogen growth due to its low pH of roughly 2.0. Pepsin is the resultant, active enzyme from pepsinogen. It cleaves proteins into smaller fractions to increase digestibility in the small intestine. The pyloric sphincter allows the passage of a small amount of chyme into the duodenum (McDowell, 2010).

1.8.2 The large and small Intestines: "lower" GIT and accessory organs

The bulk of digestion and absorption occur in the duodenum. Lacteals contained within the villi of the mucosa layer are responsible for the uptake and transport of nutrients to the body via the circulatory and lymphatic systems. Various cells contained within the villi perform specialized functions, such as enzymatic and hormonal secretion, and mucus production. Cells in the submucosa secrete alkaline mucus to neutralize the acidic chyme or any residual hydrochloric acid. The chyme is pushed along the small intestine via muscle contractions, which also helps it mix with secretions from the gallbladder, liver, and pancreas (McDowell, 2010; M. E. Smith & Morton, 2011). The liver is a large organ that is responsible for filtering metabolites and aiding digestion by the production of bile. The gallbladder, part of the liver, is a storage organ for bile, and excretes bile to help emulsify fats for subsequent breakdown and absorption. The pancreas secretes pancreatic juice, a cocktail of enzymes for the breakdown of foods. Amylase, bile, lipase, chymotrypsin, trypsin, carboxypeptidase, elastase, deoxyribonuclease, and ribonuclease are all secreted in the small intestine. Whatever is indigestible by these enzymes passes through the ileocecal valve, important for preventing contamination of the small intestine with microorganisms naturally present in the large intestine (McDowell, 2010; M. E. Smith & Morton, 2011). The large intestine is not lined with protruding villi; the surface is relatively smooth and is composed of mucosal cells that are important in regulating pH against acid producing bacteria. A large amount

of water is absorbed, along with important salts, such as sodium. While the large intestine serves its purpose before fecal excretion, it also houses many interesting symbiotic bacteria that are important for health (McDowell, 2010; M. E. Smith & Morton, 2011).

1.9 Microbial flora of the human digestive system

Commensal microorganisms densely colonize the lower human digestive system while not inducing an immune response in the host (Jandhyala et al., 2015). Approximately 7000 different microbial strains and over 35,000 species of bacteria are present in the human gut, with the human body colonized by up to 1014 microorganisms – 70% of which is found largely in the human colon (Jandhyala et al., 2015; Tabernero & Gómez de Cedrón, 2017). Bacteroides, Firmicutes, Actinobacteria, and Proteobacteria make up approximately 90% of bacterial phylotypes (Sivieri, Bassan, Peixoto, & Monti, 2017). Bacteria tend to selectively inhabit different layers of the large intestine, where luminal bacteria that are identifiable in stool samples include *Bacteroides, Bifidobacterium, Streptococcus, Clostridium, Enterococcus, Enterobacteriaceae, Lactobacillus,* and *Ruminococcus*. Bacteria identifiable in the mucosa and mucus include *Clostridium, Lactobacillus,* and *Akkermansia* (Jandhyala et al., 2015).

Proportions and types of symbiotic microorganisms that are present vary depending on the person and their lifestyle factors (Sivieri et al., 2017; Tabernero & Gómez de Cedrón, 2017). People that tend to follow a specific dietary pattern generally have a distinctive gut microbiota corresponding to their diet. For example, lower amounts of *Bacteroides spp., Bifidobacterium spp., E.* coli, and *Enterobacteriaceae* are found in those following vegan diets compared to other bacteria that grow under a high protein diet. Plant based diets tend to favour a dense and diverse microbiota with higher short chain fatty acid production and a lower Firmicutes:Bacteroidetes ratio (Healey et al., 2017). It is suggested that large amounts of *Bacteroides, Prevotella,* and *Ruminococcus* indicate a healthy colonic flora (Jandhyala et al., 2015).

1.9.1 Inhabitant species

The inhabitant bacterial species are largely anaerobic, with some falculative anaerobes, and many belong to the Firmicutes and Bacteriodes phyla. Other species may belong to the *Actinobacteria, Proteobacteria, Verrucomicrobia, Cyanobacteria, Fusobacteria, Spirochaeates*, and *Lentisphaerae* phyla. The bacterial species within these phyla all respond differently to changes in

diet; some prefer to ferment resistant starches, others cellulose, while others proliferate given a diet that is high in protein and fat and low in carbohydrates. *Ruminococcacea*, *Lachnospiraceae, Roseburia* spp., *Eurobacterium rectale*, *Bifidobacterium* spp., *Colinsella aerofaciens*, and *Prevotella* spp. are some of the bacteria that proliferate under the influence of a high carbohydrate diet, rich in indigestible carbohydrates (Louis et al., 2014).

1.9.2 Bacterial metabolites

Microorganisms produce metabolites that are absorbed by the human body at varying degrees, which then circulate the human body performing multiple functions. The bacteria in the large intestine are responsible for the production of methane, hydrogen sulfide, butyrate, propionate, and vitamins among other compounds, as a result of fermentation and anaerobic respiration (Louis et al., 2014). The production of bacterial metabolites is a function of their utilization of essential nutrients derived from carbohydrates, proteins, fats, and phenolic compounds that resist digestion, including disposed intestinal epithelial cells (Jandhyala et al., 2015; Tabernero & Gómez de Cedrón, 2017). Some bacteria are also responsible for secreting antimicrobial compounds such as mucus and anti-microbial peptides, which modulate the types and quantities of microbes (Sivieri et al., 2017). Non-starch polysaccharides are utilized by primary degraders, such as *Bacteroides*, produce smaller molecular weight carbohydrates for consumption by other symbiotic bacteria in the colon. *Bifidobacterium, Ruminococcus, Eubacterium, Lactobacillus,* and *Clostridium* are also present and ferment carbohydrates in the colon, indicating that the symbiotic relationship between colonic bacteria is important for energy production (Van Laere et al., 2000; H.-D. Yoo et al., 2012). Under the influence of a high protein diet, certain bacterial species that can tolerate high concentrations of bile acids to produce nitrogenous compounds, ammonia, polyamines, and hydrogen sulfide. While some of the fermentation products from proteins in the large intestine is desirable, over production can lead to carcinogenic effects and can damage the mucosa lining of the colon. Excess consumption of fats and alcohol can also lead to the production of reactive oxygen species and inflammation when fermented by colonic bacteria (Louis et al., 2014).

1.9.2.1 Short chain fatty acid (SCFA) production

Dietary fiber fractions and polysaccharides differ in structure and properties; therefore, fermentation rates and degree of fermentation differ. Studies are beginning to show the

functionality of the gut flora is perhaps just as or more important than composition, being the result of a diet that is well balanced with several nutrients, including dietary fiber (Brahma et al., 2017). A study by Brahma et al. (2017) demonstrated that fecal donors following a high fiber diet had remarkably higher butyrate production as a result of efficient carbohydrate fermentation, compared to those following a higher fat and animal protein diet, where fermentation end products demonstrated greater protein fermentation. It was also noted that the fermentation of different grains led to differences in fatty acid profiles. Water soluble carbohydrates are more readily fermentable than those that are insoluble. Those individuals following a low fiber diet did not demonstrate any differences in fermentability between grains, where the high fiber group did, most likely due to more efficient fermentation (Brahma et al., 2017). Healy et al. (2017) also found that production of short chain fatty acids was more efficient with a stabilization period using high fermentable carbohydrate media compared to a low fermentable carbohydrate media.

The fermentable substrate can greatly influence SCFAs profiles, as well as the production of other fermentation end products (Tabernero & Gómez de Cedrón, 2017). Hairy regions of pectin, containing neutral sugars, have been shown to produce more SCFAs compared to low methoxyl apple pectin and arabinogalactan (Gulfi, Arrigoni, & Amadò, 2007). Fructooligosaccharides have been shown to produce more gas than pectic polysaccharides, however, production of propionate and acetate production is higher in pectic polysaccharides whereas butyrate production is higher in FOS fermentation (Cantu-Jungles, Cipriani, et al., 2017; Daguet et al., 2016; Min et al., 2015). The above studies all speculate that the increased production of SCFAs by FOS is due to the more rapid fermentation compared to pectic polysaccharides. Butyrate production also increases after prolonged fermentation, suggesting the conversion of acetate to butyrate via bacteria metabolic pathways (Min et al., 2015).

1.10 Prebiotics

Prebiotics are foods indigestible to human digestive processes that encourage the growth of beneficial microorganisms in the lower digestive tract that provide health benefits for the host. For a food to hold the prebiotic claim, it must be indigestible, be a fermentation substrate for microorganisms living in the gastrointestinal tract, and encourage the growth of said microorganisms. Prebiotics also increase fecal mass, frequency, and alleviate constipation, however, consumption in excess can cause adverse effects such as gases and discomfort. The

bacteria that proliferate via prebiotics are also very important in preventing colonization of pathogenic bacterial strains in the colon. *Escherichia coli, Campylobacter, Clostridium perfringens, Clostridium difficile, Listeria,* and *Salmonella spp*. are all pathogenic microorganisms that are undesirable in the colonic environment (Anadon, Martinez-Larranaga, Ares, & Aranzazu Martinez, 2016; M. Kumar, Nagpal, Hemalatha, Yadav, & Marotta, 2016). Bifidobacterium and lactobacilli play an important role in preventing adhesion and colonization of these pathogens. Beneficial bacteria can excrete acids to lower pH, making the environment undesirable for pathogens; they can colonize areas in a competitive effort to prevent adhesion and compete for nutrients; they may secrete antimicrobial agents and improve the immune system (Anadon et al., 2016).

1.10.1 Cell wall carbohydrate digestion

In order to be considered a prebiotic, the compound must resist gastrointestinal digestion. The influence of digestion on cell wall material depends highly on the starting material and the structure of the polysaccharides and any associations they may have with other cell wall constituents, such as proteins and phenolic compounds, as well as their solubility. A cell wall polysaccharide isolate from kiwi fruit showed little change in structure after being subjected to gastrointestinal digestion, however more glucose and galacturonic acid was found in soluble fractions, suggesting acidic hydrolysis of polysaccharide regions containing cellulose or homogalacturonan (Carnachan, Bootten, Mishra, Monro, & Sims, 2012). It was also observed that pectic polysaccharides in onions extracted using conditions replicating the stomach can cause hydrolysis of galactan chains (Golovchenko, Khramova, Ovodova, Shashkov, & Ovodov, 2012). This trend was also observed by Hu et al. (2013), where a polysaccharide rich in neutral sugars isolated from *Plantago asiatica* L. seeds decreased drastically in molecular weight after gastric digestion. The low pH induced significant hydrolysis of the polysaccharide due to glycosidic bond breakage. A further decrease in molecular weight was observed after intestinal digestion, where the combination of low pH from the stomach to high salt concentration may cause the disruption of polysaccharide aggregates via polymer dissociation (Hu, Nie, Min, & Xie, 2013). Similar findings were observed regarding the effects of acid hydrolysis for a polysaccharide obtained from *Ganoderma atrum* as well as the reduction in molecular weight of pectic polysaccharides obtained from potato pulp (Ding et al., 2017; Khodaei, Fernandez, Fliss, & Karboune, 2016).

1.10.2 Prebiotic properties of cell wall polysaccharides

Indigestible dietary fiber is a major energy source for gut bacteria, largely originating from the plant cell wall with minor contributions from non-cell wall materials (gums, waxes, resins, reserve carbohydrates). Different types of dietary fibers fermented by the same bacterial inoculum have been shown to produce different end-products and changes in bacterial proportions (Tabernero & Gómez de Cedrón, 2017). Cell wall polysaccharides are being increasingly investigated for their prebiotic potential. These polysaccharides vary greatly in their structure and composition. Bacteria in the colon can utilize oligosaccharides, non-starch polysaccharides, and resistant starch as sources of energy; different sources and structures of carbohydrates have varying effects on microbial growth and short chain fatty acid production. SCFAs are essential for acidifying the colon to prevent pathogen colonization (Sivieri et al., 2017; Van Laere et al., 2000; H.-D. Yoo et al., 2012).

1.10.3 Effects of dietary patterns

Studies have shown that changes in diet have a significant impact on the species present in the bacterial flora and their relative abundance. The effects of plant based and animal based (western diets) have been investigated. Short term changes in macronutrients can lead to a noticeable change in gut bacterial flora. However, individuals with a relatively healthy and diverse flora show reduced change in bacterial diversity. In particular, people with high initial Bifidobacterium counts responded minimally to changes in dietary patterns (Healey et al., 2017). A study performed by Healy et al. (2017) demonstrated that the composition of the stabilization media (high fermentable carbohydrate vs low fermentable carbohydrate) used to grow and maintain bacteria from healthy donors influenced how the bacterial flora responded to an inulin-type prebiotic, including the production of SCFAs. There is increasing evidence that an individual's bacterial flora can respond in a drastically different way, even if both follow similar diets. In fact, microbial shifts can be observed very rapidly depending on the diet of the fecal donor. David et al. (2014) observed changes in the microbiota within one day after an animal based diet reached the colon. Microbiota returned to normal after 2 days of stopping the animal based diet and the donors resumed their typical eating habits. The animal based diet caused a more significant shift in bacterial taxonomic groups than the plant based diet, where 22 clusters changed on the animal based diet and 3 on the

plant based diet. *Prevotella* was notably reduced after animal based diet consumption by a lifetime vegetarian individual. The findings in this study demonstrated the ability of the human microbiome to change between herbivorous and carnivorous metabolism, increasing evolutionary capabilities for dietary flexibility (David et al., 2014). Dietary fiber may also aid in the transport of phenolic compounds to the large intestine, while during transit small, free phenolic compounds are absorbed by the walls of the small intestine. Phenolic compounds may be linked to lignin or are present intracellularly. Phenolic compounds that reach the large intestine, such as ellagitannins, are fermentable by gut bacteria into dibenzopyran-6-one or urolithins. Proanthocyanidin cleavage results in the release of flavan-3-ols and their catabolism into valeric acids and hydroxyphenylvalerolactones (Mosele et al., 2015; Tabernero & Gómez de Cedrón, 2017).

1.11 Conclusions

By-products generated from the food industry are an opportunity to utilize otherwise overlooked sources of cell wall components. Cranberries, in particular, are berries that rich in pectic polysaccharides and other cell wall polysaccharides, as well as phenolic compounds. The rigid cell wall entraps these components, leaving them behind after juice extraction. This presents the opportunity to utilize green, novel, extraction methods, such as microwave assisted extraction, to isolate valuable compounds that may have implications on human gastro-intestinal health. The modulation of the gastro-intestinal microflora is performed by changing fermentation substrates, ultimately leading to shifts in bacterial populations. These changes can lead to increased production bacterial metabolites, notably short-chain fatty acids, that are necessary for the metabolic processes of bacteria. A well-established and optimally-functioning gut microbiota is important for overall human health and immunity. Therefore, these sources of potentially fermentable cell wall compounds should be considered as having the potential to improve human gastro-intestinal health.

CONNECTING STATEMENT I

In chapter I, the literature review encompassed descriptions of the structure of the plant cell wall as well as of the important constituents that provided added-value to the plant cell wall material otherwise considered as waste from the food industry. The complex interactions between the human gut microflora and dietary fibers were also discussed to provide an important link between chapter II and chapter III of the present study.

Chapter II investigates the production of an extract, rich in potentially prebiotic cell wall polysaccharides and polyphenolic compounds, from cranberry pomace using microwave-assisted extraction methods. The first part investigates the efficiency of a sequential acid-alkaline extraction method as compared to singular extraction with either acid or base. The second part involves studying the effects of selected parameters (including microwave power, time, sodium hydroxide concentration), through response surface methodology, on the extract yield as well as on the carbohydrate and total polyphenolic proportions and recovery.

Results from chapter II were presented at IUFOST, *18th World Congress of Food Science and Technology, 2016.*

Davis, E., Karboune, S. (2016) Microwave extraction of phenolic compounds and pectic polysaccharides from North American cranberry *(Vaccinium macrocarpon)* cell wall material using a two-step extraction method. IUFOST, *18th World Congress of Food Science and Technology, Dublin, Ireland.*

Davis, E., Karboune, S. (2018). Investigation and optimization of microwave-assisted production of an extract enriched in cell call polysaccharides and phenolic compounds from cranberry pomace (To be submitted).

CHAPTER II.

INVESTIGATION AND OPTIMIZATION OF MICROWAVE-ASSISTED PRODUCTION OF AN EXTRACT ENRICHED IN CELL WALL POLYSACCHARIDES AND PHENOLIC COMPOUNDS FROM CRANBERRY POMACE

2.1 Abstract

Extracts from cranberry pomace (*Vaccinium macrocarpon* L.) were generated using (i) acidic, (ii) alkaline, and (iii) sequential acid-alkaline microwave-assisted extraction. Extracts were assessed for their relative proportions of total phenolic compounds and total carbohydrates, recovery of total phenolic compounds, recovery of total carbohydrates relative to the total dietary fiber portion of cranberry pomace, total uronic acids, monosaccharide profile, total yield of extract, and molecular weight. Alkaline extraction generated the lowest yields (3.37%-4.96%), suggesting that homogalacturonan represents a small fraction of the cranberry cell wall. Sequential extraction combined with the yields obtained from acid extraction generated the highest yields (12.96%-11.09%) compared to acid or alkaline extraction alone. The monosaccharide profile from the sequential extraction showed high amounts of rhamnose, arabinose, and galactose, suggesting that rhamnogalacturonan I is an important constituent of the cranberry cell wall. Optimization was conducted using the microwave-assisted sequential acid-alkaline extraction method. Optimization parameters were modeled using response surface methodology, where pomace concentration (3.33-50.00 mg/ml solvent), sodium hydroxide concentration (0-2M), time (1-6 min), and microwave power (35-80 W/g cell wall material) were varied using central composite rotatable design. Optimal extraction conditions were determined as 65 W/g cell wall material, 16.33 mg/ml, 4.73 min, and 1.51 M sodium hydroxide. Yield obtained for the optimal extraction parameters was 28.65%, where recovery of total carbohydrates was 10.56% and recovery of total phenolic compounds was 57.00%.

2.2 Introduction

Food by-products are of a high interest as sources of added-value products. In particular, the cell wall components of multiple plant materials recovered after processing, such as corn biomass, potato pulp, sugar beet pulp, grape pomace, berries, onion, orange peels and apple peels, have been investigated as potential sources of fibers, oligosaccharides and antioxidants (De Camargo et al., 2016; Hartati, Riwayati, & Subekti, 2014; Müller-Maatsch et al., 2016; S.-H. Yoo et al., 2012). On the other hand, by-products from industries using woody biomass contain significant amounts of lignocellulosic materials that have been demonstrated to be potentially valuable sources of phenolic compounds and antioxidants (Ares-Peón, Garrote, Domínguez, & Parajó, 2015; Wahyudiono, Sasaki, & Goto, 2008). However, although berry pomaces, such as grapes, acai berries, black currants, and bilberries, are composed of polyphenolic compounds and cell wall polysaccharides, most studies have mainly focused on the recovery of their polyphenolic and anthocyanin compounds as added-value products (Cantu-Jungles, Iacomini, Cipriani, & Cordeiro, 2017; Cserjési et al., 2011; Gil-Sánchez et al., 2017b; Marlett & Vollendorf, 1994). In particular, cranberry pomace attracted a high interest due to its ability to prevent urinary tract infections and to promote cardiovascular and colon health owing to their polyphenolic compounds (Feliciano, Heintz, Krueger, Vestling, & Reed, 2015; Howell et al., 2005; Kaspar & Khoo, 2013). Hence, most studies on the cranberry pomace were largely focused on characterizing and extracting their polyphenolic compounds (Ou & Gu, 2015; Vattem & Shetty, 2002; White et al., 2010). Indeed, approximately 95% of cranberries are destined for the production of juice, jams, and jellies, leaving behind a large amount of pomace as a by-product, representing up to 15% of the original mass (Česonienė & Daubaras, 2016). In addition to the polyphenolic compounds, cell wall material of cranberry pomace, rich in non-starch polysaccharides, is a potentially valuable source of prebiotic carbohydrates, making this pomace a useful starting biomass for the production of an added-value material (White et al., 2010). To the best of the authors' knowledge, no study has previously investigated the potential of co-extracting polyphenolic compounds and pectic polysaccharides from cranberry pomace.

Cell wall materials from plants, including their processing by-products, have been subjected to multiple extraction techniques ranging from traditional methods to the most recent using advanced technologies. Simple solvent extraction methods are becoming less prevalent, due to the increased efficiency, improved quality of extracts, and reduced solvent consumption when combined with

extraction techniques such as ultrasound, pulsed electric field, high-pressure, microwave, and enzymatic procedures. While novel extraction methods are increasingly investigated on a laboratory batch scale, transition to scaled-up operation is poorly understood, hampering their potential for industrial applications (Raghavan, Adetunji, & Adekunle, 2017). Microwave-assisted extraction has been explored for extracting plant cell wall components, including polysaccharides, polyphenols, and essential oils (Q. Chen, Hu, Yao, & Liang, 2016; Seixas et al., 2014; Valdes et al., 2015). The microwave extraction technique has several advantages, including reduced solvent consumption, lower energy requirements than conventional heating, reduced time, increased extraction efficiency and high quality end-products (Buranov and Mazza, 2009; Bélafi-Bakó et al., 2012; Nenadis et al., 2013; Veggi *et al.*, 2013; Seixas et al., 2014; Liew et al., 2016). However, microwave extraction efficiency may be affected by some independent parameters such as the solvent to solute ratio, extraction time, and the dielectic potential of the solvent, since microwave energy is generated via dipole rotation and ionic conduction (Raghavan et al., 2017) The influence of extraction solvent is highly important, as the use of neutral, acidic, or basic extraction conditions can lead to the isolation of various non-starch polysaccharides within the plant cell wall; many studies focused on the use of acidic extraction conditions for the extraction of homogalacturonan regions of pectin (Müller-Maatsch et al., 2016; Saulnier & Thibault, 1987; Seggiani et al., 2009; Yuliarti et al., 2015). To our knowledge, only few studies have investigated the potential for a simultaneous extraction of pectin's homogalacturonan and neutral polysaccharide regions by a sequential, acidic and alkaline microwave-assisted extraction, or by other means of green extraction technology (Khodaei & Karboune, 2013; W. Zhang et al., 2017). Moreover, no study has been published regarding the co-extraction of pectic polysaccharides and polyphenolic compounds from cranberry pomace using microwave-assisted extraction technology. The main objective of the present study was to generate a value-added extract from Québec cranberry pomace using a two-step, acidic and alkaline microwave assisted extraction. The recovery of both polysacccharides and total phenolic compounds was assessed, and the neutral polysaccharide profile and uronic acid content were determined. The optimal extraction parameters were also investigated by varying sodium hydroxide concentration (M), time (min), microwave power (W/g cell wall material), and pomace to solvent ratio (mg/ml).

2.3 Materials and Methods

All chemicals were reagent grade or HPLC grade for chromatographic analysis. NaOH was

purchased from Fisher Scientific (Waltham, MA). Anhydrous ethanol was purchased from Les Alcools de Commerce (Boucherville, QC). D-galactose, D-mannose, L-rhamnose, L-arabinose, D-xylose, D-glucose, D-galacturonic acid, 85% m-hydroxybiphenyl, sulfuric acid (95-98%, A.C.S grade), hydrochloric acid (37%, A.C.S. grade), phenol, Folin & Ciocalteu's phenol reagent, trifluoroacetic acid, sodium tetraborate, sulfamic acid (99.3%), potassium sulfamate, gallic acid, and salts were purchased from Sigma Aldrich (St. Louis, MO). Kestose, nystose, and GF4 were purchased from Wako Chemicals USA (Cedarlane Laboratories, Burlington, ON).

2.3.1 Starting material by-products

Frozen cranberry pomace by-product, recovered from the juicing process, was obtained from Canneberges Atoka Inc. (Quebec, Canada). The pomace was dried in a 45 °C oven for 24 hours and then ground using a commercial blender (Waring Pro). The dried pomace was then sieved using a #16 sieve (Canadian Standard Sieve Series), and frozen at -20 °C until needed.

2.3.2 Characterization of cranberry pomace

2.3.2.1 Determination of carbohydrate content

2.3.2.1.1 Total carbohydrate content

Total carbohydrate content of pomace was determined by using the phenol-sulfuric acid assay (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) with D-glucose as standard. Pomace samples (1 mg/ml) were suspended in water by mixing for 20 min, and 400 µl of the suspension were added to 200 µl of phenol solution (5% in water). A volume of 1000 µl of concentrated H_2SO_4 was then added to the mixtures. After 10 min of incubation at room temperatures, the absorbance of the mixtures was read at 480 and 490 nm using a DU 800 spectrophotometer (Beckman Coulter, San Ramon, USA). The total carbohydrate contents were expressed as grams glucose equivalents per gram of extract.

2.3.2.1.2. Total uronic sugars content

The sulfamate/m-hydroxydiphenol assay (Blumenkrantz & Asboe-Hansen, 1974) was used to determine the galacturonic acid content of pomace samples, using D-galacturonic acid as a standard. Pomace samples were dissolved in water (0.25-5.0 mg/ml). To 400 µl of this suspension, 40 µl of 4 M sulfamic acid-potassium sulfamate and 2400 µl of 75 mM sodium tetraborate solutions were successively added. The mixtures were incubated at 100 °C for 20 min, and then cooled to room temperature. A volume of 80-µl m-hydroxyphenyl reagent (0.15%) was then added

to the mixtures and vortexed before reading the absorbance at 525 nm with a spectrophotometer. The total uronic sugar contents were expressed as grams galacturonic acid equivalents per gram of extract.

2.3.2.1.1 Determination of total dietary fiber content

Total dietary fiber content was determined using a total dietary fiber assay kit (Sigma, Germany). A 1-g sample of dried cranberry pomace was mixed with 50 ml phosphate buffer (pH 6.0) and 100 µl heat-stable α-amylase and incubated for 15 min at 95 °C. Protease from *Bacillus licheniformis* (5 mg) was then added to mixture and incubated for 30 min at 60 °C with continuous shaking. The pH of the mixture was adjusted to pH 4.0-4.5 before addition of 100 µl of amyloglucosidase and incubation for 30 min at 60 °C with continuous shaking. Four volumes of 95% ethanol were added to the mixture which was left to precipitate overnight. Samples were filtered and dried in a vacuum oven and weighed once completely dry. Ash content was determined for the samples as well as the total nitrogen content using the Dumas method. Total dietary fiber was determined as dry precipitation residue – ash – protein.

2.3.2.1.2 Determination of monosaccharide profile

The pomace samples were first subjected to the acid hydrolysis according to the method of Khodaei & Karboune (2013). A 10-mg sample of extract was suspended in 200 µl of water, and a mixture of HCl/methanol (1:4 v/v) was added at a ratio of 1:5 (v/v). The mixtures were incubated at 70 °C for 24 hrs for hydrolysis. To the hydrolyzed mixtures, 3.0 ml of water were added along with trifluoroacetic acid (TFA) at a ratio of 1:8 (v/v). After boiling at 100 °C to evaporate the TFA (to a volume of 4000 µl), samples were neutralized with NaOH and freeze dried.

The monosaccharide profile was determined using acid-hydrolyzed extracts centrifuged at 8,000 rpm for 5 min to precipitate insoluble material. An HPAEC-PAD Dionex ICS 3000 system equipped with a Carbopac PA-20 guard column (3x 30 mm) and Carbopac PA-20 column (3 x 150 mm) at a column temperature of 30ºC was used to determine monosaccharide profile and concentrations of cranberry products. A NaOH solution (5 mM) was used as the mobile phase at a flow rate of 0.4 ml/min. Rhamnose, arabinose, glucose, xylose, galactose, and mannose were used at varying concentrations as standards. Chromeleon software was used to construct calibration curves and anaylze data.

2.3.2.2. Determination of total phenolic content

Phenolic compounds were extracted from cranberry pomace with 80% methanol in water (v/v) under high pressure using a Dionex ASE 350 accelerated solvent extractor (Thermo Fisher Scientific Inc.). The pomace samples (0.50 g) were placed in 22 ml cells containing Ottawa sand and diatomaceous earth. The samples were subjected to 3 repeated extractions at 40 °C under approximately 1600 psi to exhaust the pomace. The extracts recovered were stored at 4 °C and their phenolic contents determined using Folin assay. A 20-µl sample was added to 1580 µl water along with 100 µl Folin's reagent. A 300-µl carbonate solution (250 g/L) were then added and the mixtures were incubated at 40 °C for 30 min. Absorbance of the resulting mixtures was measured at 765 nm and the total phenolic contents were expressed as gallic acid equivalents (GAE).

2.3.2.3 Characterization of molecular weight distribution

The molecular weight distribution was determined using high performance liquid chromatography size exclusion chromatography with a refractive index detector (Waters 1525 Binary Pump) and TSK-GEL Oligo-PW, TSK-GEL PW_{XL} G3000 and TSK-GEL PW_{XL} G5000 size exclusion columns (Tosoh Bioscience, Japan). 0.1M NaCl was used as the mobile phase at a flow rate of 0.4 ml/min. Both enzymes and pomace samples were analyzed to ensure detected mono- and oligosaccharides were from reactions. Nystose, kestose, fructo-furanosyl-nystose (GF4) (Wako Pure Chemical Industries, Japan), inulin, arabinose, sucrose, and galacturonic acid were used as standards for the correlation of molecular weight and retention time.

2.3.3 Microwave-assisted extraction

The extraction procedure was performed using following three methods: (i) acidic extraction, (ii) sequential acid/alkaline extraction and (iii) alkaline extraction (Figure 2.1) A 1-g sample of pomace was placed in glass pyrex extraction chamber of Prolabo Synthewave 402 Microwave Reactor and 30 ml of 0.1M HCl were added to yield a pomace to solvent ratio of 1:30 (w/v). The microwave-assisted acidic extraction was performed at 36 or 72 W/g pomace for 4 min. The supernatants were recovered by vacuum filtration using a Whatman® 47 mm GF/D glass microfiber filter, neutralized with NaOH and precipitated with 2 volumes of anhydrous ethanol. For the sequential and the alkaline extractions, the acid treated pomace and the non-treated pomace, respectively, were suspended in 30 ml of 0.15M NaOH to yield a pomace to solvent ratio of 1:30 (w/v). The alkaline extraction was performed for 4 min at 36 or 72 W/g pomace. The recovered supernatants were neutralized and precipitated with 2 volumes ethanol.

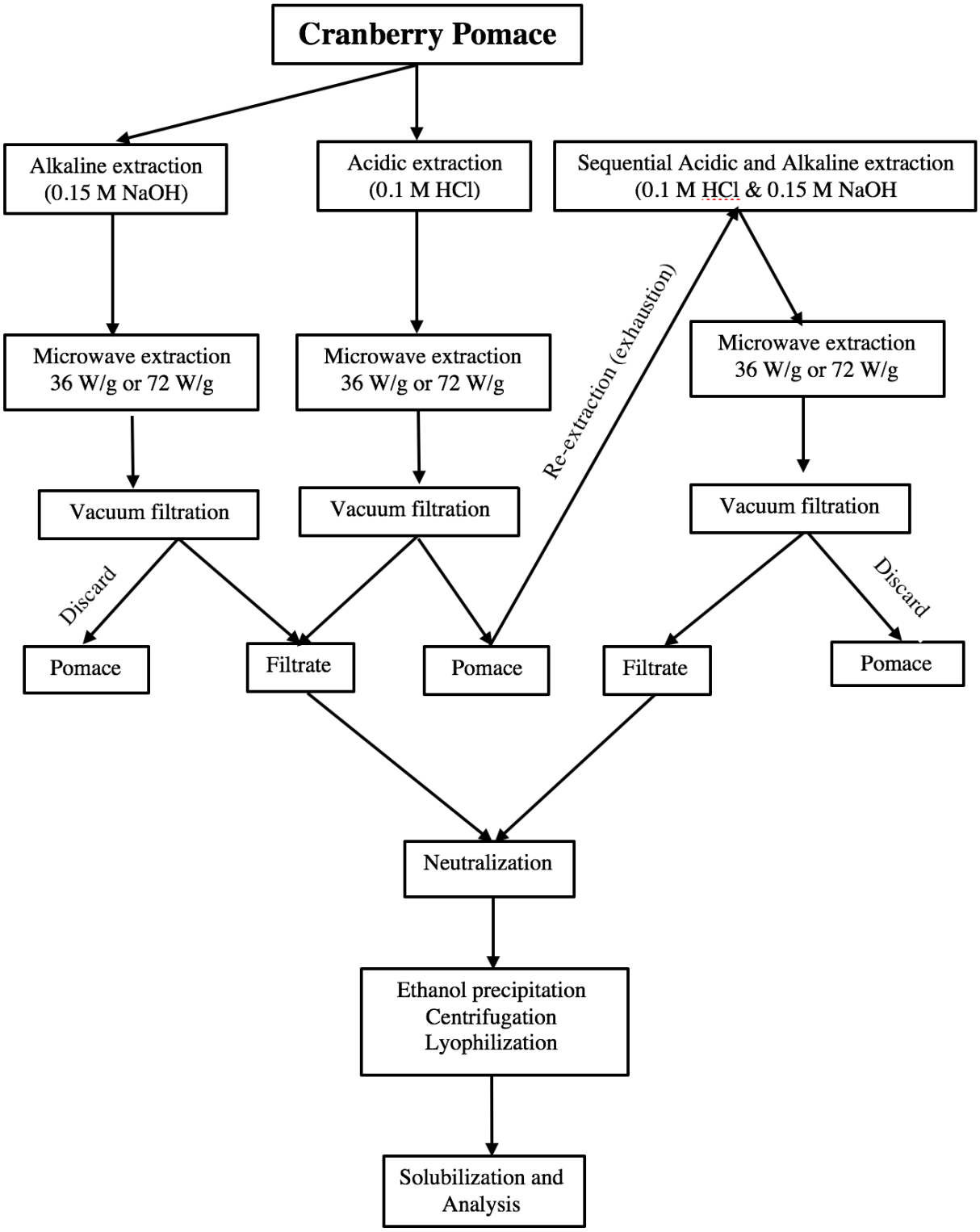

Figure 2.1. Flowsheet showing the different procedures used to prepare cranberry pomace for microwave-assisted extraction, and subsequent treatments and analysis of the extracts.

2.3.4 Experimental design for the optimization of sequential microwave-assisted extraction

Sequential microwave-assisted extractions were performed under different conditions to determine their influence on total phenolic content, total carbohydrates, total uronic acids, and extract yield using response surface methodology. Four independent variables with five levels were studied; sodium hydroxide concentration (0, 1, 0.5, 1.5, 2 M), extraction time (1, 2.25, 3.5, 4.75, 6 min), microwave power (20, 35, 50, 65, 80 W/g cell wall material), and concentration of pomace (3.33, 15.00, 26.67, 38.33, 50.00 mg pomace/ml solvent). These variables were assessed using central composite rotatable design (CCRD). The design generated 28 runs; runs were performed in duplicate; certain points were repeated in triplicate to confirm results. The center point was repeated eight times; whereas axial points were repeated four times (±α of 1.5); factorial points repeated four times (±1). Pure error was obtained from center point replicates.

2.3.5 Statistical analysis

Statistical significance and model validation was performed using analysis of variance (ANOVA). Regression coefficients for terms were used to create equations for the response surface diagrams through the Design-Expert 8.0.2 software (Stat-Ease, Inc., Minneapolis, MN, USA). A two-factor interaction equation was used to predict total phenolic content and extract yield while a quadratic equation was used to predict enrichment yield, total carbohydrates, and total phenolic content and carbohydrates (Khodaei & Karboune, 2014).

A generalized, two-factor interaction model is described by the equation below (1).

$$y_{ij} = \mu + \beta_j + \tau_i + \varepsilon_{ij} \quad (1)$$

Where μ is the mean, β_j is the *j*th block effect, τ_i is the *I* th treatment effect, ε_{ij} is the experimental error (1).

A generalized, quadratic model is described by the equation below (2).

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (2)$$

Where y is the predicted response, β_0 is a constant, β_i is the linear effect coefficient, β_{ij} the interaction effect coefficient, β_{ii} the quadratic effect coefficient, X_i and X_j are the coded level of independent variables.

Contour plots were developed by fixing independent variables constant while varying the two other variables (Khodaei & Karboune, 2014).

2.4 Results and Discussion

2.4.1 Cranberry pomace composition

Table 2.1 summarizes the composition of the cranberry pomace in terms of total phenolic content, total carbohydrate content, total dietary fiber content and the monosaccharide profile. Fresh cranberries have been reported to contain 570 mg gallic acid equivalent/100g (Česonienė & Daubaras, 2016; C. Wang & Zuo, 2011). The phenolic content of the cranberry pomace was reported to vary from 6.0 mg/g pomace to 9.8 mg/g pomace (Vattem & Shetty, 2002; White et al., 2010), suggesting that the juicing process removes a large amount of phenolic compounds. The high total phenolic content of 22.78 mg/g pomace determined in the present study can be attributed to the efficiency of high-pressure extraction method used and/or to the juice extraction method, which may have been less efficient in removing the total phenolic compounds leaving a large part of these compounds in the pomace. Also, White et *al.* (2010) determined the dietary fiber content to be 61.2 g/100g of pomace, consistent with the results of 63.04 g/100g pomace found in the present study for this constituent.

The monosaccharide profile of Table 2.1 shows that neutral sugar-based polysaccharides (60.45%) are predominant in the cranberry pomace compared with uronic acid-based polysaccharides (39.54%). The neutral sugar profile is composed of 20.45% of mannose/xylose, 14.61% of glucose, 12.13 % of arabinose and 10.50 % of galactose revealing the predominance of hemicellulose in cranberry cell wall; this is similar to what was shown for pear pomace (Rabetafika et al., 2014). In addition, the relatively low proportion of rhamnose (2.77%) compared to arabinose (12.13%) and galactose (10.50%) suggests that the rhamnogalacturonan I region of cranberry pectin is branched with arabinan and galactan chains. The rhamnogalacturonan I region of potato pectin was also reported to be highly branched with galactan where galactose represented 52.8 – 67.4% of the constitutive monosaccharides (Khodaei & Karboune, 2013). In contrast, the rhamnogalacturonan I region of pectin from apple pectin is relatively small, and the pectic polysaccharide is largely homogalacturonan, as indicated by the Gal A representing 70-94% of the monosaccharide moieties (Silva, Nunes, Coimbra, & Guido, 2014).

Table 2.1 Total phenolic content, neutral sugar and uronic acid proportions, and neutral monosaccharide profile of cranberry pomace.

Compounds	Content
Total Phenolic Content[a] (mg/g)	22.78 (± 1.50)
Dietary Fiber[b] (g/100g)	63.04 (± 2.84)
Non-dietary Fiber[b] (g/100g)	22.12 (± 1.21)
Monosaccharide profile (% w/w)[c]	
- Uronic acid	39.54 (± 0.64)
- Rhamnose	2.77 (± 0.33)
- Arabinose	12.13 (± 0.89)
- Galactose	10.50 (± 0.86)
- Glucose	14.61 (± 0.93)
- Xylose/Mannose	20.45 (± 0.63)

[a]Determined as gallic acid equivalents (GAE) per gram of pomace.

[b]Determined per dry weight of pomace.

[c]Relative proportion of pomace (%, w/w).

Citrus peel pectins from orange, lemon, lime, and grapefruit contained 24.08%, 24.33%, 29.74%, and 25.91% Gal A, respectively (Kaya *et al.*, 2014). According to the same study, rhamnose varied from 1.14 - 1.54%, arabinose from 6.66% - 8.87%, glucose from 16.57-20.51%, and galactose from 2.87 – 5.89% for orange, lemon, lime, and grapefruit peel pectin (Kaya, Sousa, Crépeau, Sørensen, & Ralet, 2014a). Black and red currant press cake pectins contained 37.1 and 49.7% Gal A respectively, which is similar to the uronic acid content determined for cranberry pomace in the present study. Similar proportions were determined for xylose, mannose, glucose and galactose, while the arabinose content of the black and red currant press cakes were lower (5.7% and 2.3%, respectively) than the that of 12.13% found in cranberry pomace (Cserjési et al., 2011). The cranberry pomace analyzed in this study was used as a starting material for the production of a value-added extract enriched with polysaccharides and polyphenols.

2.4.2. Production of Extracts Rich in Polysaccharides and Polyphenolic Compounds

Selected microwave-assisted extraction methods (acid, alkaline, sequential acid/alkaline) at 32 and 72 W/g pomace were investigated for the production of extracts enriched with oligo/polysaccharides and polyphenolic compounds. The yield, the recovery of the polyphenolic compounds and the recovery of cell wall carbohydrates are depicted in Table 2.2. Overall, the yields of extract generated at 36 W/g pomace were higher than those obtained at 72 W/g pomace. Acidic, alkaline, and sequential acidic/alkaline generated 4.96%, 12.65%, and 8.00% at 36 W/g, respectively, and 3.37%, 10.36%, and 7.72% at 72 W/g, respectively. This can be attributed to the fact that higher microwave energy generates greater solvent heating, which may have resulted in more extensive breakdown of plant polysaccharides into mono- and disaccharides, thereby reducing the overall yield. Indeed, the recovered extracts were separated from the hydrolysed compounds by ethanol precipitation. Similar results were reported for the microwave extracted pectin from mango peel, where microwave power in excess of 48 W/g caused decreased yields (Maran, Swathi, Jeevitha, Jayalakshmi, & Ashvini, 2015). Similarly, Dong *et al*. (2016b) observed that polysaccharide yield extracted from *Chuanminshen violaceum* roots decreased at microwave powers above 500W/g corresponding to a maximum yield of 12.84%. High extract yield at low powers implied that total carbohydrate recovery would be highest at the low power of 36 W/g, which can be seen from Table 2.2. Carbohydrate recoveries at 36 W/g was 2.65%, 7.84%, and 5.27% for acidic, alkaline, and sequential acidic/alkaline extraction, respectively, compared to corresponding carbohydrate recoveries at 72 W/g of 2.35%, 4.73%, and 4.20%, respectively. We

anticipate, therefore, that increasing microwave energy leads to increased breakdown of cell wall material, which in turn reduces the recovery of total carbohydrates with a negative impact on extract yield. Total phenolic recovery followed a similar trend, where higher recovery occurred at lower microwave power. Acidic, alkaline, and sequential acidic/alkaline treatments recovered 13.21%, 100.0%, and 52.22% total phenolic compounds. Ester bonds of polyphenolic compounds are broken-down under high temperature conditions, which can be directly related to the increased microwave power (Buranov & Mazza, 2009; Nenadis et al., 2013). For instance, the production of phenolic compounds from corn auto-hydrolysis liquors was found to decrease with increasing temperature, demonstrating the degradative effect of high temperature on phenolic compounds (Ares-Peón et al., 2015). Recovery of total phenolic compounds decreased to 4.06%, 51.76%, and 51.06% for acidic, alkaline, and sequential treatments. As stated above, it can be confirmed that the recovery of carbohydrates had the most prominent effect on the extract yield through the relative proportions of total carbohydrates and phenolic compounds. As expected, the carbohydrate proportions of all extracts were higher than the phenolic compound proportions (Table 2.2) due to the carbohydrate content representing a larger portion of cranberry pomace than the total phenolic compounds (Table 2.1).

The lowest yields of 4.96% and 3.37% at 36 and 72 W/g, respectively, were obtained by the acidic extraction. Plant cell wall materials extracted at low pH were shown to result mainly in the recovery of homogalacturonan regions of pectin via protopectin hydrolysis (Yeoh, Shi, & Langrish, 2008), and to cause cleavage of neutral sugar chains via ester bond hydrolysis (Khodaei & Karboune, 2013). Sugar beet pectin extracted with hot hydrochloric acid between pH 1.0 and pH 2.0 and a solid to liquid ratio of 1:20 (w/v) generated yields between 6.16% to 24.96% (Lv, Wang, Wang, Li, & Adhikari, 2013). As compared to the cranberry pomace, the higher yields obtained upon acidic extraction of sugar beet pectin may be attributed to its high homogalacturonan content, which can range from 35.2 - 76.3% of the total weight of pectic polysaccharides, depending on acid concentration, temperature, and extraction time (Yapo, Robert, Etienne, Wathelet, & Paquot, 2007). Acidic extraction generated the lowest recovery of total carbohydrates (2.35-2.65%) and total phenolic compounds (4.06-13.21%), which explains the low extract yields obtained. Low carbohydrate recovery suggests that cranberry contains fewer homogalacturonan regions compared to other sources of pectic polysaccharides, such as sugar beet.

Table 2.2. Production of extracts enriched with cell wall carbohydrate and polyphenolic compounds using acidic, alkaline and sequential acidic/alkaline microwave-assisted extraction methods at two selected microwave power levels.

	Acid Extraction		Alkaline Extraction		Sequential Extraction	
	36 W/g[e]	72 W/g	36 W/g	72 W/g	36 W/g	72 W/g
Yield (% w/w)[a]	4.96 (± 0.34)	3.37 (± 0.98)	12.65 (± 2.63)	10.36 (± 0.02)	8.00 (± 0.91)	7.72 (± 1.49)
Total Carbohydrate Recovery (% w/w)[b,c]	2.65 (± 0.001)	2.35 (± 0.012)	7.84 (±0.013)	4.73 (± 0.002)	5.27 (±0.01)	4.20 (± 0.023)
Total Phenolic Compound Recovery (% w/w)[b]	13.21 (± 1.37)	4.06 (± 0.51)	100.0 (± 14.74)	51.76 (± 5.64)	52.22 (± 6.33)	51.06 (± 7.14)
Relative Proportion of Carbohydrates (%)[d]	84.96	93.76	69.84	71.67	73.63	69.34
Relative Proportion of Phenolic Compounds (%)[d]	15.04	6.24	30.16	28.33	26.37	30.66

[a]Yield calculated as amount of pomace ÷ amount of pomace × 100. [b]Relative to the amount determined in original pomace. [c]Recovery of total carbohydrates in the extract relative the total amount of dietary fiber in original pomace. [d]Relative proportion of the sum of both total phenolic compounds and carbohydrates in the extract. [e]Watts per gram of cell wall material (pomace).

Poor extraction of phenolic compounds using acidic extraction was also reported by Waszkowiak *et al*. (2015) who demonstrated that acid hydrolysis of flaxseed extracts produced lower total phenolic compounds compared to alkaline hydrolysis. Because acidic media are able to isolate pectin via protopectin hydrolysis (Yeoh et al., 2008) but are unable to extract phenolic compounds, acidic extraction produced cranberry extracts with higher proportions of carbohydrates than phenolic compounds. Indeed, this can be seen in Table 2.2 showing the high carbohydrate proportions of 84.96% and 93.76% at 36 W/g and 72 W/g, respectively, and relative proportions of 15.04% and 6.24% total phenolic compounds at 36 W/g and 72 W/g, respectively.

Both alkaline extraction and sequential acidic/alkaline extraction generated yields higher than acidic extraction. Alkaline conditions can cause hydrolysis of galacturonic acid chains, preserving neutral sugar regions of pectin (Yeoh et al., 2008). These results suggest neutral sugar regions of pectin, such as rhamnogalacturonan I, are an important component of the pectic polysaccharides in cranberry. One explanation of higher yield obtained with alkaline extraction media would be the isolation of lignin and hemicellulosic polysaccharides. A study using sequential extraction on black currant pomace generated 6.32 mg/g and 92.8 mg/g for dilute alkali and concentrated alkali extraction, where the authors suggest that the high yield of alkali soluble polysaccharides is due to high content of hemicellulose in the pomace (Kosmala et al., 2010). In our study, the alkaline extraction generated yields of 12.65% and 10.36% at 36 W/g and 72 W/g respectively, while the yields from the sequential acidic/alkaline extraction were 8.00% and 7.72%, for 36 W/g and 72 W/g, respectively (Table 2.2). The lower yields obtained through sequential extraction are possibly due to the initial acidic extraction performed on the pomace. This may have significantly hydrolyzed neutral sugar regions of pectin, causing them to remain soluble in the ethanol/solvent mixture during alcohol precipitation (Dong, Zhang, et al., 2016). The greater extract yields of the two treatments involving alkaline media would imply greater recovery of total carbohydrates and/or total phenolic compounds.

Alkaline and sequential acidic/alkaline extraction generated higher total carbohydrate recoveries than acidic extraction as well as higher total phenolic compound recovery. Indeed, the dietary fiber profile of cranberries is rich in hemicellulose and lignin, leading to increased carbohydrate recovery using alkaline conditions compared to acidic. Carbohydrate recovery for sequential acidic/alkaline extraction (4.20-5.27%) was lower compared to alkaline (4.73-7.84%). It is

expected that the first acid extraction step removed homogalacturonan, which would reduce the overall carbohydrate recovery of a sequential alkaline extraction. Along with the first acidic extraction, tissue softening would have occurred, encouraging more leaching of carbohydrates into the solvent leading to greater breakdown of polysaccharides (Lefsih et al., 2017), which may explain the lower recovery of carbohydrates by the sequential extraction than the alkaline extraction. The total phenolic compounds' recovery was higher for both treatments involving alkali, contributing to the higher yields obtained with the alkaline methods. According to Bouras *et al.* (2015), greater amounts of total phenolic compounds were produced at higher pH values than under acidic conditions. The authors attributed this to ester bond hydrolysis of polyphenolic compounds, such as tannins and phenolic acids, and to the degradation of lignin and cell wall damage. Alkaline extraction at 36 W/g produced the greatest phenolic recovery, at 100.0%, and 51.75% at 72 W/g. We express recovery as relative to the initial amount in the starting material (pomace). We hypothesize that the high recovery is due to the liberation of phenolic compounds entrapped in the cell wall that were not completely extracted using the ASE method used on the pomace. The sequential alkaline extraction generated 52.22% recovery at 36W/g, and a similar recovery of 51.0% at 72 W/g. A comprehensive study determined the total phenolic content of multiple plant materials, including berries, using traditional solvent extraction method. Small cranberries, red currants, black currants, and strawberries had total phenolic contents of 21.1, 12.6, 20.3, 23.7 mg GAE /g dry weight, respectively (Ka, 1999). The total phenolic contents determined in the present study for the extracts generated using 36 W/g were 2.87, 11.90, and 21.23 mg GAE/g pomace for acidic, sequential, and alkaline extraction, respectively (data not shown). The alkaline extraction generated total phenolic content similar to that of small cranberries, black currants, and cranberries, whereas the sequential extraction was similar to the content determined for red currants using traditional solvent extraction (Ka, 1999). This demonstrates that microwave extraction is an efficient means to extract phenolic compounds compared to traditional solvent methods. The use of alkaline media to isolate phenolic compounds was also proven to be efficient through the relative proportion of these compounds in the extracts, as the proportion of total phenolic compounds was higher for alkaline (28.33-30.16%) and sequential acid/alkaline extraction (26.37-30.66%) compared to acidic extraction (6.24-15.04%).

2.4.3 Characterization of the cranberry recovered extracts

2.4.3.1 Monosaccharide Profile

The cranberry extracts generated using acidic, alkaline, and sequential acidic/alkaline microwave-assisted extraction were characterised for their neutral sugar and uronic acid contents as well as their monosaccharide profiles (Table 2.3). The proportions of each fraction varied according to the microwave power: mild (36 W/g pomace) or high (72 W/g pomace). Acidic and alkaline microwave-assisted extractions produced carbohydrate extracts with similar uronic acid (55.9%) and neutral sugar (44.5%) proportions at 36 W/g; while a higher proportion of uronic acid (60.0%) was obtained by the sequential acidic/alkaline extraction. It has been reported that dilute alkali can further extract ester bound pectins, hence increasing the total amount of homogalacturonan extracted (Hilz, Bakx, Schols, & Voragen, 2005). The results of Table 2.3 show that higher microwave power of 72 W/g increased the uronic acid-rich carbohydrates recovered by acidic and sequential acidic/alkaline extractions to 60.5% and 64.0%, respectively. In contrast, higher proportion of carbohydrates rich in neutral sugars (50.5%) was obtained by alkaline extraction at 72 W/g. These results can be explained by the higher susceptibility of homogalacturonan region of pectic polysaccharides to breakdown via β-elimination at intense microwave power conditions under alkaline conditions (Barrett, Athon, & Diaz, 2007) or by the higher proportion of hemicellulose extracted at higher microwave power; indeed, alkaline conditions promote the isolation of hemicelluloses (Oosterveld, Beldman, Schols, & Voragen, 2000). Compared to the findings on berry pectins reported by Müller-Maatsch *et al.* (2016), the contents of uronic acids obtained for the cranberry in the present study were lower than that reported for berry pectin isolated using a chelator, where berries extracted using a chelator generated pectic polysaccharides containing 0.744 g uronic acid/g pectin. However, berry pectin extracted by using dilute alkali without a chelator contained 0.596 g uronic acid /g pectin, which is similar to the proportion extracted from cranberries at 36 W/g pomace (60.06% uronic acids) (Müller-Maatsch et al., 2016). These results may be attributed to the lower uronic acid content of cranberries than the other berries and/or to the difference in the extraction efficiency.

Table 2.3 summarizes the results on the monosaccharide profile of neutral sugar portion of cranberry extracts obtained by different microwave-assisted methods at 36 W/g or 72 W/g. The Table shows that all extraction methods at 72 W/g yielded a higher rhamnose proportion in the neutral sugars, with the highest proportion (4.15%) recorded in the acidic extraction, whereas

similar proportions of this monosaccharide, 3.96% and 3.76%, were obtained by the alkaline and sequential extraction methods, respectively. These data suggest that acidic extraction is an important step when the objective is to isolate pectic polysaccharides rich in rhamnogalacturonan I, a pectic polysaccharide composed of an alternating backbone of D-galacturonic acid and L-rhamnose with neutral sugar side chains of arabinose and galactose (Khodaei & Karboune, 2013).

Table 2.3. Uronic acid and neutral sugar proportion of generated cranberry extracts using various microwave assisted extraction methods at 36 W/g or 71 W/g, and the monosaccharide profile of the neutral sugar portion.

Extraction method	Uronic Acids (%)[a]	Neutral Sugars (%)[a]	Rhamnose (%)[b]	Arabinose (%)[b]	Galactose (%)[b]	Glucose (%)[b]	Xylose + Mannose (%)[b]
				36 W/g			
Acid Extraction[c]	55.95 (± 5.23)[f]	44.05	3.00 (± 0.32)	17.02 (± 1.13)	10.82 (± 0.52)	8.66 (± 0.87)	5.36 (± 0.24)
Alkaline Extraction[d]	55.99 (± 15.21)	44.01	2.23 (± 0.25)	14.66 (± 0.78)	10.03 (± 0.79)	10.81 (± 0.97)	5.78 (± 0.46)
Sequential Extraction[e]	60.06 (±2.38)	39.94	2.40 (± 0.20)	15.91 (±0.67)	12.53 (± 0.32)	5.09 (± 0.58)	4.20 (± 0.46)
				72 W/g			
Acid Extraction[c]	60.50 (± 3.95)	39.5	4.15 (± 0.16)	9.89 (± 0.35)	11.68 (± 0.32)	8.39 (± 0.56)	5.30 (± 0.52)
Alkaline Extraction[d]	49.53 (± 10.87)	50.47	3.96 (± 0.21)	20.81 (± 1.80)	14.75 (± 0.44)	5.45 (± 0.66)	4.87 (± 0.40)
Sequential Extraction	64.02 (± 8.39)	35.98	3.76 (± 0.27)	11.10 (± 0.71)	13.87 (± 0.65)	3.78 (± 0.25)	3.75 (± 0.43)

[a]Proportion of total carbohydrate content (% w/w). [b]Relative proportion of total neutral sugar content (% w/w). [c]0.1M HCl, 3.33 mg pomace/ml HCl, 4 min. [d]0.1M NaOH, 3.33 mg pomace/ml NaOH, 4 min. [e] 0.1M HCl, 3.33 mg pomace/ml HCl, 4 min followed by 0.1M NaOH, 4 min. [f]Standard deviation denoted in brackets

Arabinose proportions in the neutral sugars, with microwave-assisted extraction at 36 W/g, were 17.02, 14.66 and 15.91% for acidic, alkaline, and sequential extractions, respectively. Significantly different proportion of arabinose were obtained by these extraction methods, when the microwave-assisted method was run at a power of 72 W/g. In particular, alkaline extraction generated neutral sugars with less arabinose than did the acidic and sequential extractions. Indeed, the arabinose proportions were 20.81, 9.89 and 11.10% for the alkaline, the acidic and sequential extraction methods, respectively. Acidic conditions are known to cause the breakdown of arabinofuranosyl linkages thereby degrading polysaccharides containing arabinose, such as rhamnogalacturonan I or arabinogalactans (Yuliarti et al., 2015). The increase of temperature generated at 72 W/g during microwave-assisted extraction may have exacerbated the breakdown of arbinan chains induced under acidic conditions, whereas these chains are more protected under alkaline conditions. The neutral sugars extracted at 72 W/g also contained higher proportions of galactose than those extracted at 36 W/g. Then proportion of this monosaccharide in the neutral sugars extracted with the former method were 11.68, 14.75, and 13.87% for acidic, alkaline, and sequential extraction, respectively. It can be speculated that the higher is the microwave power, the more pectic polysaccharides can be obtained. Furthermore, the fact that galactose is a constituent of rhamnogalacturonan I, may explain why acidic and sequential extractions generated greater amounts of galactose than alkaline extraction via the hydrolysis of these polysaccharides from the homogalacturonan backbone (Moore et al., 2008).

The presence of glucose indicates the presence of glucans in the extracts, including cellulose and (1,3)/(1,4)-β-glucan. These polysaccharides can be degraded via the acid hydrolysis by the combination of high heat and acidic conditions, thereby releasing glucose moieties (Lee et al., 2015). Kiwi fruit pomace extracted with acid produced polysaccharides containing glucose, as a result of the cellulose breakdown of (Yuliarti et al., 2015). The results of Table 2.3 show that alkaline extraction generated the highest proportion of glucose (10.81%) at 36 W/g. Alkaline conditions tend to favor the extraction of hemicellulose which is linked to cellulose, suggesting that some cellulose can be extracted in the process (Saulnier and Thibault, 1987; Yuan et al., 2016). At 72 W/g, acidic extraction generated the greater proportion of glucose (8.96%) compared to alkaline (5.45%) and sequential (3.78%) extraction, indicating that the hydrolysis of cellulose polymers increases with increased microwave energy. Xylose/Mannose remained relatively constant for all extraction methods, varying in proportion from 3.75% to 5.78% between 36 W/g

and 72 W/g. Xylose and mannose are indicative of hemicellulose extraction (Yapo et al., 2007); compared to the xylose/mannose proportion of cranberry pomace (20.45%, Table 2.1) the proportion extracted using acidic, alkaline, and sequential treatments is lower. Hence, xylose and mannose were not fully extracted or hemicellulose was not extensively degraded under the conditions used in the present study.

The study performed by Müller-Maatsch et al. (2016) found that mixed berry by-products contained relative proportions of 87.5% uronic acid, 3.82% arabinose, 2.05% rhamnose, 4.43% galactose, and 2.2% glucose in the total pectin extracted with dilute alkali. The same authors reported that chelator-soluble pectic polysaccharides isolated from berry by-products contained 80.3% uronic acid, 4.32% arabinose, 1.51% rhamnose, 10.0% galactose, and 3.88% glucose. The relative proportions of uronic acid in the dilute alkali soluble and chelator soluble berry pectin was higher than that isolated from cranberry pomace (49.53-64.02%; Table 2.3). However, proportions of rhamnose and arabinose isolated from cranberry pomace (2.23-4.15% and 9.89-20.81%, respectively) were higher than the berry pectins isolated by Müller-Maatsch et al. (2016). Glucose content was similar for the cranberry extract generated using 72 W/g sequential acidic alkaline extraction (3.75%). Galactose proportion was high in berry pectins, as reported by Müller-Maatsch et al., (2016), as well as in the cranberry polysaccharide extract studied herein. This suggests that differences in starting material and method of extraction yield extracts with polysaccharides of highly different nature and monosaccharide profile. It should be mentioned, however, that our results are in agreement with those of Maatsch et al., (2016) on berry fruits regarding the high proportions of arabinose and galactose in berry cell wall polysaccharides, suggesting that rhamnogalacturonan I is a major polysaccharide in pectin. Pectic polysaccharide isolated from tangerine peels was shown to be composed of 42.5% uronic acid, 23% arabinose, 20% galactose, 6.9% rhamnose, 4.2% glucose, and 3.5% mannose (Chen et al., 2016b). Uronic acid content was lower than that determined for cranberries, while arabinose, galactose, and rhamnose content was higher, indicating that the rhamnogalacturonan I region is more prominent in citrus pectin, as shown by Chen et al (2016b). Glucose proportion of the pectic polysaccharides isolated from cranberry pomace were higher than tangerine peel (4.2%) under all conditions except sequential acidic/alkaline extraction at 72 W/g, suggesting that cellulose content is lower in citrus peels than in cranberry pomace. Apple pectin extracted using acidic conditions was reported to generate on average, 50.8% uronic acid, 4.77% arabinose, 0.92% rhamnose, 3.71% galactose, 26.8% glucose,

and 6.64% xylose/mannose (Sato et al., 2011). The uronic acid content of the apple pectin reported by Sato et al., (2011) is similar to that determined for cranberry pectin in this study, whereas the glucose proportion was higher in apple pectin. Conversely, rhamnose and arabinose proportions were higher in pectin extracted from cranberry than from apple, suggesting that rhamnogalacturonan I is more abundant in the former pectin extract.

Table 2.4 shows the galacturonic acid and monosaccharide ratios as an insight to the pectin structure of cranberries. The extract obtained with microwave-assisted isolations at 36 W/g yielded polysaccharides with high GalA/Rha ratios, with the highest ratios (25:1) obtained upon the alkaline and the sequential acid/alkaline extractions. These results suggest that homogalacturonan chains are preserved at lower microwave energy. They also indicate that pectic polysaccharides may have been released due to their de-esterification from other plant cell wall polysaccharides, such as lignin and hemicellulose. Compared with results from other studies, the GalA/Rha ratios determined in cranberry pectic polysaccharides were lower than those reported for apple pectin (73:1, 67.8:1) and berry by-product pectin (42.6-51.3:1), higher than those obtained for citrus pectin (6.2:1), and similar to those reported for pectin isolated from black currant pomace (15.5-24:1) (Kosmala et al., 2010; Sato et al., 2011; Wang and Lü, 2014; Chen et al., 2016b; Müller-Maatsch et al., 2016). The overall results suggest that pectins isolated from cranberry pomace are composed of shorter homogalacturonan chains and a high number of rhamnogalacturonan I regions. The degree of branching of rhamnogalacturonan I can be predicted through the galactose and the arabinose to rhamnose ratios. Berry press cake pectins showed Ara:Rha and Gal:Rha of 1.9-2.9:1 and 2.2-6.6:1, respectively, while apple pectin was on average 6.2:1 Ara:Rha and 4:1 Gal:Rha (Sato et al., 2011; Müller-Maatsch et al., 2016). At 36 W/g pomace for all extraction methods, the Ara:Rha (5.7-6.6:1) and Gal:Rha (3.6-5.2:1) ratios were closer to those of apple pectin. Increasing microwave power to 72 W/g reduced the ratios, indicating hydrolysis of the arabinan and galactan chains of rhamnogalacturonan I. The sequential acid/alkaline extraction at 36 W/g, yielded the highest branching of arabinan chains (6.6:1) and galactan chains (5.2:1), whereas alkaline extraction at 72 W/g generated the highest branching of arabinan (3.7:1) and galactan (5.3:1). The Gal/Ara shows that at 36 W/g, arabinan chains predominate over galactan chains within rhamnogalacturonan I. However, at 72 W/g, acid extraction and sequential extraction generated slightly more galactan branches.

Table 2.4. Galacturonic acid and neutral monosaccharide ratios for different extracts generated using microwave assisted extraction of cranberry pomace.

Extraction method	Gal A:Rha[a]	Gal:Rha	Ara:Rha	Gal:Ara
		36 W/g		
Acid Extraction	18.7	3.6	5.7	0.6
Alkaline Extraction	25.1	4.5	6.6	0.7
Sequential Extraction	25.0	5.2	6.6	0.8
		72 W/g		
Acid Extraction	14.6	2.8	2.4	1.2
Alkaline Extraction	12.5	3.7	5.3	0.7
Sequential Extraction	17.0	3.7	2.9	1.2

[a]Gal, Ara, Rha, Gal A correspond to galactose, arabinose, rhamnose, and galacturonic acid, respectively.

2.4.3.2. Molecular Weight Distribution

The results on the molecular weight distribution of the polysaccharides in the extracts generated by using acidic, sequential, and alkaline microwave-assisted extraction are depicted in Figure 2.2. Varying microwave power caused changes in molecular weight distribution of carbohydrate, and this was particularly evident for acidic extraction. Interestingly, higher microwave power (72 W/g) extraction under acidic conditions released extracts with higher proportion of high molecular weight polysaccharides; up to 70% of the total polysaccharides released had MW in the 200-700 kDa range. A possible explanation is that high power induces severe tissue softening, cell rupture, and cell component leakage, which facilitate the extraction of large polysaccharide chains (Lefsih et al., 2017).

For all the methods of extraction at 36 W/g, polysaccharides with higher proportions of low MW (1-10 kDa) were extracted, suggesting that these small molecular weight compounds are significantly affected by higher microwave energy. The 1-10 kDa population, from 36 W/g to 72 W/g, decreased from 28.9% to 7.6% for acidic extraction, 21.8% to 5.7% for sequential acid/alkaline extraction, and from 16.1% to 12.0% for alkaline extraction. The proportion of low MW polysaccharides was less affected by increasing microwave power with alkaline extraction, implying that acidic extraction has a hydrolytic effect on the pectic polysaccharides in cranberry, which adds up to the effect of the microwave high energy. Proportions of polysaccharides in the 10-120 kDa range at 36 W/g were 48.9%, 59.0%, and 70.3% for acidic, sequential acidic/alkaline, and alkaline extraction, respectively. This population of polysaccharides was favoured under alkaline conditions. At 72 W/g, acidic, sequential acidic/alkaline, and alkaline extraction generated 25.9%, 53.9%, and 42.1%, respectively, of polysaccharides in the 10-120 kDa range. All three extractions demonstrated a reduction in the relative proportion of this size distribution, implying the occurrence of a hydrolytic effect at higher microwave power. Polysaccharides in the 120-200 kDa range were only obtained in acidic extraction at 36 W/g, at a proportion of 22.2%. Higher microwave power caused hydrolysis of the polysaccharide that was only generated under acidic conditions. Polysaccharides in the 200-700 kDa range at 36 W/g were absent in the acidic extraction, but present at 19.2% and 13.6% in sequential acidic/alkaline extraction and alkaline extraction, respectively.

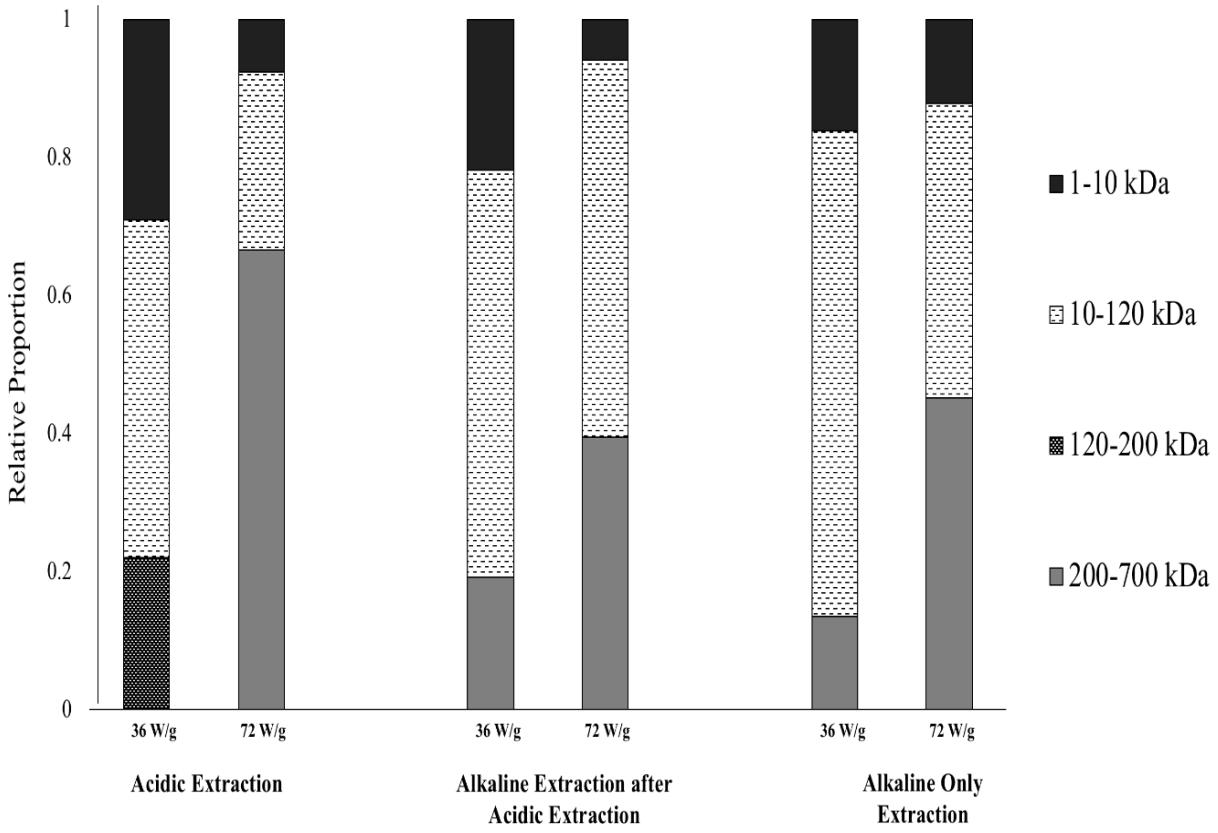

Figure 2.2. Molecular weight distribution of carbohydrates extracted from cranberry pomace using acid, sequential, and alkaline microwave assisted extraction methods, determined using HPLC-SEC, expressed as relative proportion.

At 72 W/g, these high MW polysaccharides were detected at acidic, sequential acidic/alkaline, and alkaline extractions at the respective proportions of 66.5%, 39.4%, and 44.9%, respectively. These results indicate that the higher microwave power was necessary to generate larger molecular weight polysaccharides, possibly through tissue softening and increased liberation of cell wall components. The microwave extraction of passion fruit peels using weak organic acids (1:25 g/ml passion fruit peel pulp, 356-628 W) by Seixas *et al.* (2014) yielded pectins ranging between 496 and 229 kDa, which is similar to those extracted in the present study. *Chuanminshen violaceum* roots extracted using microwave assisted extraction generated pectic polysaccharides with a size ranging between 2 and 406 kDa (Dong, Lin, et al., 2016; Dong, Zhang, et al., 2016). Similarly, pumpkin biomass produced microwave extracted polysaccharides (pH 2.5; solid to liquid ratio of 30-50:1, 1200 W, 2-6 min) in the range of 41 to 469 kDa (Košťálová, Aguedo, & Hromádková, 2016). These studies demonstrate that microwave extraction can generate a diverse molecular weight profile depending on the conditions and starting material, and also confirm that the polysaccharides extracted using all methods in the present study are within a comparable range for plant biomass isolation using microwave-assisted extraction.

2.4.4 Optimization of two-step, sequential microwave-assisted extraction method

The successful sequential microwave-assisted extraction method allows the generation of extract enriched with both phenolic compounds and large molecular weight polysaccharides containing both galacturonic acid and neutral sugars. Further optimization of this extraction process for the valorization of cranberry pomace was carried out. Response surface methodology (RSM) was used to investigate the effects of sodium hydroxide concentration (x_1 – 0-2M), microwave power (x_2 – 35-80 W/g cell wall material), amount of cranberry pomace (x_3 – 3.33-50 mg pomace/ml solvent), and time (x_4 – 1-6 min) on the total phenolic content, purity, yield, total carbohydrates, and enrichment yield obtained during microwave extraction. The range of extraction parameters was determined after preliminary trials. The investigated pomace (solid) to solvent ratio was similar to that reported by Košťálová et al. (2016) and Lefsih et al. (2017), in which solid to solvent ratios ranged from 20:1 - 33.3:1 mg/ml and 40:1-100:1 mg/ml, respectively. Hartati et al. (2014) used very low microwave power (6 W/g watermelon rind), whereas other studies applied more aggressive treatment with much higher microwave power, ranging from 100-480 W/g cell wall material (Košťálová et al., 2016; C. S. Kumar et al., 2016; Lefsih et al., 2017; Maran et al., 2015). The present study investigated milder extraction power at 36 and 72 W/g pomace for acidic,

sequential acidic/alkaline, and alkaline extraction solvents than the above-mentioned studies, which did not investigate the two-step sequential acid-alkaline method. The investigated extraction time was within the same range as those (1-3 min) reported by Lefsih et *al*. (2017). Preliminary trials were carried out in order to determine an appropriate range of NaOH for optimization with respect to carbohydrate recovery, total phenolic content, and yield.

Table 2.6 summarizes the extraction parameters investigated for each run along with the responses. Treatment n°24 (1.5 M, 35 W/g, 15 mg/ml, 2.25 min) produced an extract with the highest total phenolic recovery, with 95.98% recovery. The low solid to liquid ratio most likely provided a better concentration gradient for the diffusion of phenolic compounds into the solvent, thereby increasing the total recovery. Phenolic compounds are also better extracted under more alkaline conditions, due to the ester bond hydrolysis from the plant cell wall. The low microwave power and short extraction time may have caused less extensive phenolic compound degradation compared to higher power and longer extraction time. Treatment n°1 (0.0 M, 50 W/g, 26.67 mg/ml, 3.5 min) produced the lowest total carbohydrate recovery (3.26%), phenolic compound recovery (11.06%), and the lowest extract yield (4.46%) This treatment confirms that the acidic pre-treatment (0.1 M HCl) is necessary to induce cell tissue softening by the heat generated via microwave treatment, ultimately opening up the cell wall network (Lefsih et al., 2017). The use of low concentration HCl (0.1M) encourages the release of neutral polysaccharides by improving ß-elimination of homogalacturonan (Vian & Chemat, 2014).The higher yields and better recovery observed after the alkaline extraction treatments indicate that the sequential alkaline extraction is an important process to exhaust the cranberry cell wall material of the pectin neutral carbohydrate regions and the de-esterification of phenolic compounds from cell wall polysaccharides. Treatment n°20 (1.5 M, 65 W/g, 15 mg/ml, 4.75 min) gave the highest yield (29.86%) with highest recovery of carbohydrates (17.58%) and fairly good recovery of phenolic compounds (75.18%). The high yield can be attributed to the low solid to liquid ratio, which increases the diffusion rate of the target compounds into the solvent, facilitating extraction and generating higher yields. Moderate microwave power and prolonged time would have helped to exhaust the pomace of the target compounds, without causing extensive hydrolysis and losses. The high carbohydrate recovery is an indication that these conditions are favourable for the generation of cranberry cell wall polysaccharides.

Table 2.6. Experimental conditions and responses generated using Design Expert® for extract yield, proportion of total phenolic content and carbohydrates, recovery of phenolic compounds, and recovery of carbohydrates.

Run	x_1 - NaOH (M)	x_2 - Power (W)[a]	x_3 – Pomace (mg/ml)[b]	x_4 - Time (min)	Extract Yield (%)[c]	Total Phenolic Compound Proportion[d]	Carbohydrate Proportion[d]	Recovery of Phenolic Compounds (%)[e]	Recovery of Carbohydrates (%)[e,d]
1	0	50	26.67	3.50	4.46	10.91	89.09	11.06	3.26
2	0.5	65	38.33	2.25	10.97	20.14	79.86	47.45	6.80
3	0.5	65	15.00	4.75	15.10	13.32	86.68	33.99	7.99
4	0.5	65	15.00	2.25	13.97	7.97	92.03	19.99	8.34
5	0.5	35	38.33	2.25	9.59	11.64	88.36	22.14	6.07
6	0.5	35	15.00	2.25	12.37	9.19	90.81	16.23	5.80
7	0.5	35	38.33	4.75	11.85	25.30	74.70	58.47	6.24
8	0.5	35	15.00	4.75	13.43	15.48	84.52	31.48	6.21
9	0.5	65	38.33	4.75	14.58	28.27	71.73	79.58	7.30
10	1	50	26.67	1.00	16.26	10.96	89.04	34.88	10.24
11	1	50	26.67	3.50	16.23	21.34	78.66	69.65	9.28
12	1	50	26.67	3.50	17.43	20.07	79.93	60.87	8.76
13	1	50	3.33	3.50	0.00	-	-	-	-
14	1	50	50.00	3.50	14.95	20.79	79.21	66.29	9.13
15	1	20	26.67	3.50	14.69	16.05	83.95	41.86	7.91
16	1	50	26.67	3.50	21.86	20.47	79.53	56.31	7.90
17	1	50	26.67	6.00	18.36	21.01	78.99	87.61	11.90
18	1	50	26.67	3.50	17.25	16.64	83.36	39.76	7.20
19	1	80	26.67	3.50	16.82	22.88	77.12	77.78	9.47
20	1.5	65	15.00	4.75	29.86	13.39	86.61	75.18	17.58
21	1.5	65	38.33	2.25	17.77	16.38	83.62	72.57	13.39
22	1.5	35	15.00	4.75	22.04	12.98	87.02	42.72	10.35
23	1.5	35	38.33	4.75	20.65	7.12	92.88	33.15	15.63
24	1.5	35	15.00	2.25	26.60	20.50	79.50	95.98	13.45
25	1.5	65	15.00	2.25	27.27	11.91	88.09	52.21	13.95
26	1.5	35	38.33	2.25	18.72	12.42	87.58	53.15	13.54
27	1.5	65	38.33	4.75	20.21	21.29	78.71	83.15	11.11
28	2	50	26.67	3.50	26.47	17.27	82.73	83.01	14.37

[a] Power was W/g of pomace (dry weight).[b] mg/ml of both 0.1M HCl and NaOH. [c] % (w/w) with respect to starting amount of pomace.[d] Relative proportion to the sum of both carbohydrates and phenolic compounds contained in the extract. [e] Relative to the amount present in pomace. [d] Calculated relative to the amount of total dietary fiber content of pomace.

2.4.4.1 Analysis of variance

The statistical significance of the mathematical models used for the modeling of experimental results (yield, proportions of phenolic compounds and carbohydrates, recovery of phenolic compounds and total carbohydrates) as a function of extraction parameters (NaOH concentration, microwave power, pomace concentration, and time) was determined using analysis of variance (ANOVA) (Table 2.7, α=0.05). Significance was more specifically determined by comparing *F* and *P* values, lack of fit, and coefficient R^2. The linear model was the most statistically significant for both extract yield and recovery of total carbohydrates with coefficient R^2 of 0.89 and 0.87 and *F* value of 37.03 and 27.34, respectively. Both responses had *P* value of <0.0001, indicating that the *F* values obtained for the models have less than a 0.01% probability of being obtained due to noise. Lack of fit was determined as insignificant relative to pure error, with *F* value of 0.43 and 1.39 for yield and recovery of total carbohydrates, respectively. The proportion of phenolic compounds and carbohydrates both followed a statistically significant quadratic model with coefficient R^2 of 0.96 for both and *F* value of 15.57 and 12.98, respectively. *P* values of <0.0001 and <0.0005, indicating that the *F* values obtained for the models have less than a 0.01% ad 0.05% probability of being obtained due to noise for the proportion of total phenolic compounds and carbohydrates, respectively. Lack of fit relative to pure error was insignificant for proportion of total phenolic compounds and carbohydrates, with *F* value of 0.49 and 0.57, respectively. Lastly, a two-factor interaction (2FI) model was statistically significant for the recovery of phenolic compounds, with coefficient R^2 of 0.9, *F* value of 9.71 and *P* value of 0.0003. Lack of fit was insignificant relative to pure error (*F* value of 0.51).

Extraction parameters and their interactions were examined using the *F* and *P* values (Table 2.7). Regarding linear effects, sodium hydroxide concentration ($\boldsymbol{x}_1$) was the most significant variable for yield with an *F* value of 131.95 and *P* value <0.0001 with pomace concentration ($\boldsymbol{x}_3$) as the second most significant variable with *F* value of 15.39 and *P* value of 0.001. According to equation (3), an increase in sodium hydroxide (+) and a decrease in pomace concentration (-) would predict higher yield. Sodium hydroxide concentration was also the most significant linear variable for recovery of total phenolic compounds and recovery of total carbohydrates ($\boldsymbol{x}_1$), with *F* value of 29.29 and 91.20 and *P* value of 0.0002 and <0.0001, respectively.

Table 2.7. Analysis of variance for the response surface model for extract yield, proportion of phenolic compounds, proportion of total carbohydrates, recovery of phenolic compounds, and recovery of total carbohydrates.

Source	Extract Yield (%)		Proportion of Phenolic Compounds (% w/w)		Proportion of Carbohydrates (% w/w)		Recovery of Phenolic Compounds (%)		Recovery of Total Carbohydrates (%)	
	F Value	*P* Value	*F* Value	*P* Value	*F* Value	*P* Value	*F* Value	*P* Value	*F* Value	*P* Value
Model	37.03	< 0.0001	15.57	0.0001	12.98	0.0005	9.71	0.0003	27.34	< 0.0001
x_1- NaOH (M)	131.95	< 0.0001	3.35	0.1006	4.08	0.0780	29.29	0.0002	91.20	< 0.0001
x_2- Power (W/g)	5.43	0.0316	6.05	0.0362	2.70	0.1391	16.88	0.0015	4.58	0.0471
x_3- Pomace (mg/ml)	15.39	0.0010	106.00	< 0.0001	83.30	< 0.0001	3.30	0.0943	0.4100	0.5305
x_4- Time (min)	0.4870	0.4942	39.28	0.0001	41.20	0.0002	2.39	0.1481	4.69	0.0449
$x_1 \cdot x_2$	-	-	0.1340	0.7228	1.92	0.2035	1.55	0.2364	-	-
$x_1 \cdot x_3$	-	-	44.15	< 0.0001	38.19	0.0003	8.02	0.0151	-	-
$x_1 \cdot x_4$	-	-	52.89	< 0.0001	34.36	0.0004	9.92	0.0084	-	-
$x_2 \cdot x_3$	-	-	55.16	< 0.0001	35.55	0.0003	1.60	0.2302	-	-
$x_2 \cdot x_4$	-	-	10.69	0.0097	13.35	0.0065	0.8402	0.3774	-	-
$x_3 \cdot x_4$	-	-	5.97	0.0371	8.46	0.0196	2.62	0.1317	-	-
x_1^2	-	-	30.59	0.0004	0.5366	0.4848	-	-	-	-
x_2^2	-	-	0.0595	0.8128	0.2704	0.6171	-	-	-	-
x_3^2	-	-	14.25	0.0044	18.62	0.0026	-	-	-	-
x_4^2	-	-	5.07	0.0508	8.28	0.0206	-	-	-	-
Lack of Fit	0.4246	0.8870	0.4892	0.7907	0.5732	0.7266	0.5134	0.8075	1.39	0.4426

$P \leq 0.05$ indicates statistical significance.

R^2 = 0.8676, 0.8986, 0.8840, 0.8338 for extract yield, proportion of phenolic compounds, proportion of carbohydrates, recovery of phenolic compounds, and recovery of total carbohydrates, respectively.

Both equations (6) and (7) show a positive relationship between sodium hydroxide concentration and recovery of total phenolic compounds and total carbohydrates. Alkaline pH encourages ester bond hydrolysis and the release of phenolic compounds as well as carbohydrates (Buranov & Mazza, 2009). The proportion of phenolic compounds and carbohydrates had pomace concentration ($\boldsymbol{x}_3$) as the most significant linear variable, with F values of 106 and 83.3, respectively, and P<0.0001 for both. An increase in pomace concentration results in an increase in the proportion total phenolic compounds (Eq 4), whereas a decrease in pomace concentration leads to an increase in the proportion of total carbohydrates (Eq 5). The two-factor interaction between sodium hydroxide ($\boldsymbol{x}_1$) and time ($\boldsymbol{x}_4$) was the most significant variable interaction for recovery of total phenolic compounds (F value of 9.92 and P value of 0.0084). The interaction between these two variables has an antagonistic effect on the recovery of total phenolic compounds. This may be due to prolonged exposure time leading to increased heating and decomposition of thermally labile phenolic compounds (Karaaslan et al., 2014). Interactions between sodium hydroxide ($\boldsymbol{x}_1$) and time ($\boldsymbol{x}_4$) (F value of 52.89; P<0.0001) and power ($\boldsymbol{x}_2$) and pomace concentration ($\boldsymbol{x}_3$) (F value of 55.16; P<0.0001). The most significant quadratic effect was sodium hydroxide ($\boldsymbol{x}_1^2$) with F value of 30.59 and P value of 0.0004. Similar to the effect of the variable interactions on the recovery of total phenolic compounds (Eq 6), the proportion of total phenolic compounds decreases as a result of the interaction between time and sodium hydroxide concentration ($x_1\,x_4$; Eq 4). However, the interaction between pomace and power has a positive effect on the recovery of total phenolic compounds ($x_2\,x_3$; Eq 4). Two factor interactions for the proportion of total carbohydrates were all statistically significant. The most statistically significant variable interaction occurred between sodium hydroxide and pomace ($x_1\,x_3$) with an F value of 38.19 and P value of 0.0003; the equation (5) indicates that the interaction of these two variables has a positive effect on the proportion of carbohydrates. In contrast, the most significant linear variable interaction demonstrates that an increase in pomace (x_3) concentration contributes to a decrease in yield. The most significant quadratic interaction for this response was also the pomace concentration (x_3^2). Given that the recovery and proportion of total carbohydrates is related, the increase in recovery with increasing sodium hydroxide also implies that the proportion of total carbohydrates would increase as well, which is demonstrated through the positive relationship between the two-factor variable interaction of sodium hydroxide and pomace.

Extract Yield (% w/w) = $18.29+9.85\,x_1+1.53\,x_2-3.40\,x_3$ (3)

Relative Proportion of Total Phenolic Compounds (% w/w) = $20.21+1.94\,x_2+11.25\,x_3+6.81\,x_4-13.99\,x_1\,x_3-15.21\,x_1\,x_4+11.73\,x_2\,x_3+5.13\,x_2\,x_4+5.23\,x_3\,x_4-10.00\,x_1^2-5.34\,x_3^2$ (4)

Relative Proportion of Total Carbohydrates (% w/w) = $79.91-12.20\,x_3-7.59\,x_4+17.38x_1x_3+18.80\,x_1\,x_4-14.44x_2\,x_3-7.65\,x_2\,x_4-8.48\,x_3\,x_4+6.47\,x_3^2+4.25\,x_4^2$ (5)

Recovery of Total Phenolic Compounds (% w/w) = $57.85+34.34\,x_1+14.91\,x_2-37.57\,x_1\,x_3-39.74\,x_1\,x_4+7.84\,x_2\,x_4+20.55\,x_3\,x_4$ (6)

Recovery of Total Carbohydrates (% w/w) = $9.52+5.50\,x_1+0.7275\,x_2-1.11\,x_4$ (7)

2.4.5. Effects of extraction parameters

2.4.5.1 Extract yield

It can be seen clearly from Figure. 2.2 A that increased molarity of sodium hydroxide and reduced solid to solvent ratios results in a greater extract yield, consistent with the results of previous studies (Hosseini, Khodaiyan, & Yarmand, 2015; Khodaei, Karboune, & Orsat, 2016; Košťálová et al., 2016; Prakash Maran et al., 2014). Solid to solvent ratios have been reported to have a significant influence on the yield of microwave-extracted polysaccharides via heat and mass transfer (Dahmoune, Nayak, Moussi, Remini, & Madani, 2015; Košťálová et al., 2016; Maran et al., 2015). Decreasing solid to solvent ratios allows more effectively the absorption and dispersion of microwave energy to the plant materials as heat, and also provides greater surface contact of liquid with solid particles (Q. Chen et al., 2016; C. S. Kumar et al., 2016; Maran et al., 2015). However, these studies have shown that optimal solid to solvent ratios vary significantly depending on the type of plant material used. For example, optimal pectic polysaccharide yield was reached at a solid to solvent ratios of 50:1 mg/ml and 100:1 mg/ml for mango peel (Maran et al., 2015) and berry (Bélafi-Bakó et al., 2012), respectively. Furthermore, the respective optimal ratios of 25:1 mg/ml, 125:1 mg/ml and 20:1 mg/ml were reported for the extraction of polysaccharide petcin from tangerine (R. Chen et al., 2016), watermelon rind (Hartati et al., 2014), and tamarillo and pumpkin (Košťálová et al., 2016; Y. Kumar & Belorkar, 2015), respectively. The optimal pomace to solvent ratio determined for the microwave treatment of cranberry pomace using acidic/alkaline was 16.33:1 mg/ml, which is less than the studies reported above. The starting

material (cranberry pomace) was very light and had a low weight to volume ratio, which explains the low solid to solvent ratio used.

Increasing sodium hydroxide concentration improved the extract yield. Alkaline extraction has been used to extract the neutral sugar-rich polysaccharide regions of pectin from sugar beet and potato cell walls, due to the degradation of homogalacturonan via β-elimination and oxidative peeling upon these alkaline conditions (Zykwinska et al., 2006). This is indicative that the hydrolysis of homogalacturonan favors the release of the neutral sugar regions of pectin, such as rhamnogalacturonan I and hemicelluloses. It can be hypothesized that the increase in yield that paralleled the increase in sodium hydroxide concentration is possibly due to improved extraction of neutral sugar-rich cell wall polysaccharides in cranberry pomace. This demonstrates that cell wall polysaccharides rich in neutral sugars are an important constituent of cranberry pectin, as it was reported (Bouaziz, Koubaa, Ellouz Ghorbel, & Ellouz Chaabouni, 2017; Chylinska, Szymanska-Chargot, & Zdunek, 2016; Fishman, Chau, Cooke, Yadav, & Hotchkiss, 2009).

2.4.5.2. Recovery & relative proportions of phenolic compounds and carbohydrates

Figure 2.3 D shows the contour plot generated for the recovery of total phenolic compounds relative to the original content of the pomace. Microwave power and sodium hydroxide concentration had the most statistically significant interactions regarding the recovery of phenolic compounds. Increased concentration of sodium hydroxide and higher microwave power generated the highest concentrations of phenolic compounds. Increased microwave power causes increased heating of the solvent due to accelerated molecular agitation (increase in entropy), which in turn promotes cell destruction and facilitates the leaching of phenolic compounds into the solvent (Veggi et al., 2013). The combination of high temperatures, generated at high microwave power, and the acidic/alkaline solvent may have induced the breakdown of lignin, ultimately leading to the release phenolic compounds (Bobleter, 1994; Bunzel, Seiler, & Steinhart, 2005; Wahyudiono et al., 2008). The de-esterification of phenolic compounds attached to the cell wall polysaccharides may have also been accelerated at high microwave power. The extraction of polyphenols from leaves of *Myrtus Communus* (L.) was also demonstrated to increase with the microwave power; however, it was reported that very high microwave power eventually reduces recovery rate of these compounds (Dahmoune et al., 2015). The total phenolic content generated from *Citrus Sinensis* peels also increased with increasing microwave power of up to 500 W, with the authors attributing

the increase in phenolic compounds to the breakdown of larger polyphenols (Nayak et al., 2015). However, these studies investigated microwave power up to 900W; it is possible that the range of microwave power used in the present study was not high enough to cause significant breakdown of phenolic compounds, and hence reduced recovery (Sahin, 2015). The increase in phenolic compound recovery with increasing concentrations of sodium hydroxide may be attributed to ester bond hydrolysis of polyphenolic compounds, including those esterified to cell wall components, under alkaline conditions (Bouras et al., 2015; Waszkowiak, Gliszczyńska-Świgło, Barthet, & Skręty, 2015). Increased concentration of sodium hydroxide would also change the dielectric properties of the solvent; increased polarity implies greater molecular movement and increased heating, and therefore more efficient extraction of target compounds (Li, Fabiano-Tixier, Abert-Vian, & Chemat, 2013; Setyaningsih, Saputro, Palma, & Barroso, 2015).

The recovery of carbohydrates of the total dietary fiber content (Figure 2.3 E) was also affected by the sodium hydroxide concentration and the microwave power. High sodium hydroxide concentration was effective at maximizing the carbohydrate recovery, regardless of microwave power. Typically, higher microwave power tends to generate more cell wall breakdown and leaching of cell wall constituents into the solvent (C. S. Kumar et al., 2016). The non-significant effect of the microwave power may be due to the high resistant of polysaccharides extracted under alkaline conditions to breakdown compared to acid soluble or water-soluble cell wall polysaccharides (Coll-Almela et al., 2015; Fishman et al., 2009).

Contour plots B and C of Figure 2.3 show the proportions of phenolic compounds and total carbohydrates in the extract, respectively. Both responses were significantly affected by the extraction time and the pomace concentration. However, the proportion of phenolic compounds increased with the time of extraction and the pomace concentration, while the proportion of carbohydrates was higher at low pomace concentration, regardless of the extraction time. However, the total phenolic content is significantly lower than the total carbohydrate content in the cranberry pomace (22.78 mg GAE/g pomace vs. 85.16 g CHO/100g pomace). The higher proportions of carbohydrate are largely due to their extraction from cell wall where they are major constituents. Large concentration of pomace generated high proportion of carbohydrates at short exposure time. Longer extraction time was shown to result in increased heating of the extraction solvent and promotes cell rupture (Prakash Maran et al., 2014).

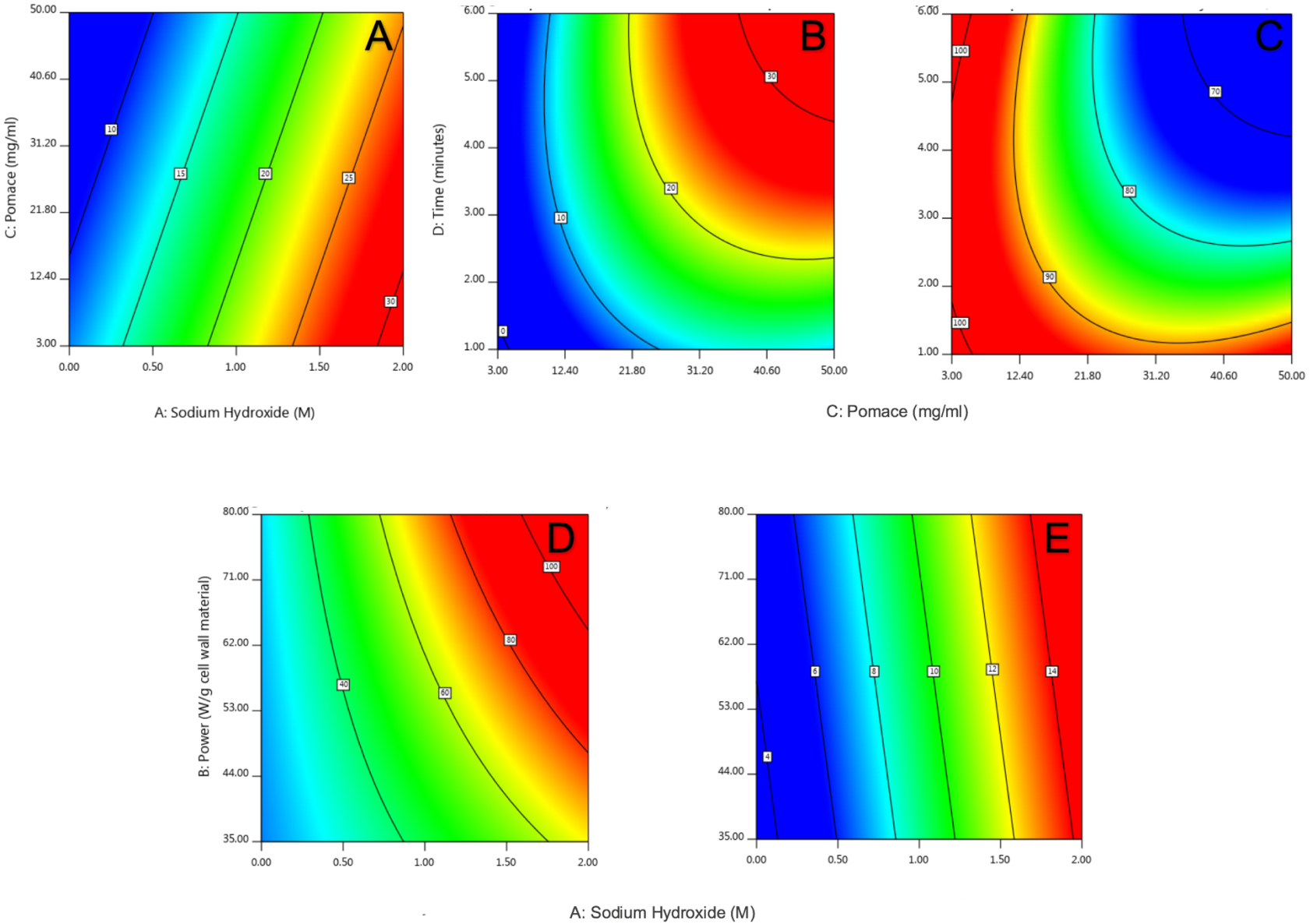

Figure 2.3 A-E. Contour plots of responses generated using response surface methodology of extracts generated using sequential microwave assisted extraction of cranberry pomace. A: Yield of extract (% w/w); B: Proportion of phenolic compounds (%); C: Proportion of total carbohydrates (%); D: Recovery of phenolic compounds (% w/w); D: Recovery of total carbohydrates (% w/w).

The increased breakdown of cell wall material may have increased the release of phenolic compounds and carbohydrates into the solvent, but could eventually decrease the total phenolic content since these compounds are generally heat labile and heating is directly related to irradiation time (Bouras et al., 2015). Sahin (2015) observed that increased irradiation time during solvent-free microwave extraction increased the total phenolic content extracted form mandarin leaves, up to a certain point, after which total phenolic content started decreasing. However, both total phenolic compounds and carbohydrates seem to be unaffected by the maximum exposure time in the present study. Higher pomace concentration yielded a greater proportion of phenolic compounds; greater amount starting material would have provided the potential for a greater final concentration of phenolic compounds in the extract. It can also be assumed that the larger quantity of pomace has provided an isolating effect, preventing over-heating of the solvent providing a protective effect for phenolic compound to preserve their structure. Likewise, this would imply that it takes longer for the solvent to heat, making extraction at short exposure time inefficient. The opposite effect is observed for the carbohydrate proportion, implying that the extraction of both of these compounds from cranberry pomace is a sort of inverse relationship regarding exposure time and pomace concentration. Since cranberry pomace is richer in carbohydrates than in phenolic compounds (White et al., 2010), It can be speculated that high pomace concentration leads to saturation of the solvent with carbohydrates, with a concomitant reduction in the rate of transfer of target compounds into the solvent. Similarly, for the phenolic proportion, high pomace concentration may have interfered with energy transfer to the solvent, reducing heating and hence the extraction efficiency.

2.4.6 Validation of the models

Based on the predictive models, three optimal treatments were generated based on most desirable results for a specific parameter (Table 2.8). Treatment n°1 (maximum yield) consisted of 1.51 M NaOH, 65 W/g pomace, 16.33 mg pomace/ml solvent, and 4.73 min. Treatment n°2 (maximum phenolic compound recovery) involved shorter extraction time (2.25 min), lower power and higher solid to solvent ratio (35.33 W/g pomace and 38.67 mg pomace/ml solvent) and the same concentration of 1.51 M NaOH. Treatment n°3 (maximum carbohydrate recovery) was similar to treatment n°1 but had lower NaOH concentration (1.11 M) and the same pomace concentration as treatment n°2 (38.67 mg pomace/ml). These recovered extracts were further characterized for their neutral and uronic sugar profile as well as their molecular weight distribution. The treatment n°1

corresponding to the lowest concentration of pomace resulted in the highest extract yield. The predicted range for the extract yield varied from 21.20-29.90% (Table 2.8), where the experimentally obtained yield of 28.65% was within the predicted range. Treatments n°2 and n°3 generated yields (17.90% and 14.97%, respectively) within their predicted ranges of 15.55%-24.0% and 14.21-22.57%, respectively. The recovery of total carbohydrates relative to the total dietary fiber of the pomace was within the predicted ranges for all three treatments, indicating the model fit well for all three treatments. Experimentally, extraction n°1 generated the highest recovery of total carbohydrates (10.56%), which may be attributed to the low pomace to solvent ratio, long exposure time, and high microwave power. The recovery of total phenolic compounds for treatment n°1 (57.00%) and treatment n°2 (61.12%) was within the respective predicted ranges of 38.72-100% and 21.14-77.36%. Treatment n°3 generated a total phenolic recovery (30.86%) below the predicted range (57.1-100.0%). The highest recovery of phenolic compounds was obtained for extraction n°2, which has shorter extraction time compared to treatments n°1 and n°3. Overall, treatment n°1 resulted in experimental values that fit best with the predicted models.

The relative proportion of phenolic compounds obtained from treatment n°1was 16.43% (Table 2.8) whereas treatment n°2 (18.85%) generated the highest proportion of phenolic compounds and treatment n°3 generated the lowest proportion of phenolic compounds (14.40%). This suggests that short time and lower extraction power had greater influence on the extraction or preservation of phenolic compounds. Treatments n°1, n°2, and n°3 generated total carbohydrate proportions of 83.57%, 81.15%, and 85.60%, respectively (Table 2.8). Longer extraction times and higher power seem to favour greater proportions of carbohydrates via increased cell wall breakdown, as was observed for conditions n°1 and n°3.

Size exclusion chromatography was performed to characterise the molecular weight distribution of the generated polysaccharides. Figure 2.4 shows that all extracts contain a small proportion of carbohydrates between 0.5-2.5 kDa. Treatment n°2 contained the highest proportion of oligosaccharides and oligomers (16.45%) compared to treatments n°1 and n°3 (5.03% and 6.61%, respectively). Large molecular weight polysaccharides (400-900 kDa) were also highest for treatment n°2 (32.05%) compared to n°1 (12.05) and n°3 (24.71%). Lower power and short time distinguish treatment n°2 from the other treatments, suggesting that smaller molecular weight oligosaccharides and large molecular weight polysaccharides are better preserved under these conditions. The molecular weight distribution of 5-15 kDa was not detected among the

polysaccharides generated form treatment n°2, but was found in extracts generated from treatments n°1 and n°3 at the respective proportions of 23.58% and 46.64%. The higher power and longer extraction time for these two treatments may cause more extensive hydrolysis of cell wall polysaccharides compared to the conditions for treatment n°2. Treatment n°2 had the same NaOH concentration (1.51M) to treatment n°1, suggesting that longer extraction time and higher power accelerate polysaccharide breakdown under strong alkaline solvent. All three treatments generated polysaccharides in the 50-150 kDa range; however treatment n°3 contained the smallest proportion (34.46%) compared to n°1 (45.64%) and n°2 (52.9%). The shorter extraction time and lower microwave power of treatment n°2 may have prevented hydrolysis of polysaccharides within this range. Treatment n°3 had a higher proportion of 400-900 kDa polysaccharides (24.71%) compared to treatment n°1 (12.05%). Treatment n°3 had lower NaOH concentration (1.1M) but higher pomace to solvent concentration (38.67 mg/ml) than n°1, but with the same extraction time (4.73 min) and power (65 W/g). The low solid to solvent ratio of treatment n°1 (16.33 mg/ml) implies a higher rate of diffusion of carbohydrates into the solvent, leading to prolonged exposure to microwave energy, resulting in hydrolysis of polysaccharides. Combined with low pomace to solvent ratio, higher NaOH concentration would also accelerate the ß-elimination homogalacturonan (A. Zykwinska, Rondeau-Mouro, Garnier, Thibault, & Ralet, 2006). This could explain the lower proportion of large molecular weight polysaccharides in the extract generated from treatment n°1. Regardless of differences in molecular weight distributions, the data presented suggests that the optimized conditions do not cause extensive hydrolysis of generated oligo- and polysaccharides.

Table 2.8. summarizes the percentage of neutral and uronic sugars present in each optimal extract. Treatment n°1 generated the highest percentage of neutral sugars at 61.9% with 38.10% of uronic sugars. The uronic acid content results from the extraction of homogalacturonan ,while the neutral sugar content results from the extraction of other pectic polysaccharides, such as rhamnogalacturonan I and II, hemicellulose and lignin. As treatment n°1 consisted of longer time of exposure, high power and low pomace concentration, it is more likely that it resulted in an extensive hydrolysis of the homogalacturonan region releasing lower uronic acid content compared to the other two treatments. Zykwinska et al., (2006) have also reported that homogalacturonan regions are more sensitive to alkaline hydrolysis compared to neutral sugars rich rhamnogalacturonan region. As compared to treatment n°1, increasing the pomace

concentration to 38.66 mg/ml in treatment n°3 led to the generation of carbohydrates with a neutral sugar content of 52.4% and a uronic acid content of 47.59%, suggesting a slight removal of homogalacturonan regions. However, both treatments n°2 and n°3 resulted comparable neutral sugar profile (51.40% and 52.41%, respectively). These results reveal that the same patterns of neutral sugars-rich carbohydrates were released from treatments n°2 and n°3 with similar concentration of pomace (35.33 mg/ml). They also suggest that the use of lower power (35.3 w/g) and shorter extraction time (2.25 min) at treatment n°2 led to a neutral sugar content of 51.40% and a uronic sugar content of 48.6%. The percentage of rhamnose (Table 2.8) was markedly higher in the extract generated by treatment n°2 than in those obtained in treatments n°1 and n°3. The proportion of rhamnose generated under the conditions of treatment n°2 was 4.50% compared to 2.51% and 1.48% generated from conditions n°1 and n°3, respectively. The extraction conditions for n°2 were milder, as it used a lower microwave power and shorter extraction time. It is possible that these conditions resulted in a moderate hydrolysis of rhamnogalacturonan I. The longer extraction times and higher microwave power for treatments n°1 and n°3 could have caused extensive polysaccharides hydrolysis, resulting in the generation of monosaccharides that would not remain in the extract during the ethanol precipitation stage. Arabinose, is another constituent of the RGI region of pectin, which was discovered in very similar proportion regardless of extraction conditions, as treatments n°1, n°2, and n°3 containing the respective proportions of 13.85%, 11.61%, and 11.89%. The bonds between arabinose molecules in the arabinan chains found in RGI may resist hydrolysis by alkaline and heat. Treatment n°1 with high microwave power and NaOH concentration, and low solid to solvent ratio would allow more heat to be generated with a consequent increase in mass transfer of the target analytes into the solvent. Galactose is also present in RGI as linear or branched chains of galactan. As was the case for rhamnose, galactose was present in higher proportion in the extract generated from condition n°2 where it represented 13.78% of the neutral sugar profile. Treatments n°1 and n°3 produced extracts containing 11.07% and 8.60%, respectively (Table 2.8). As speculated with rhamnose, the milder extraction conditions potentially preserve the galactan chains, whereas increased time and power cause the hydrolysis of galactan polymers to monosaccharides. The detection of both galactose and arabinose could indicate the presence of arabinogalactan. Arabinogalactan type I consists of a backbone of β-D-(1→4) Gal with α-L- Ara or arabinan branches attached to O-3 of Gal residues, whereas arabinogalactan type II has a backbone of β-D-(1→3) Gal with β-6-linked galactan or

arabinogalactan branches (Mohnen, 2008). Glucose was present as 15.02%, 10.41%, and 13.25% of the total neutral monosaccharides for conditions n°1, n°2, and n°3, respectively (Table 2.8) This monosaccharide would originate from either hemicellulose or cellulose. Yet, the presence of xylose and mannose suggests that it is more likely to have been released from hemicellulose rather than from cellulose. Similar to glucose, xylose and mannose were present in higher proportion from conditions n°1 and n°3 at 19.56% and 17.10%, respectively and 11.10% from condition n°2. Hemicellulose is often associated with pectin in the plant cell wall, leading to their simultaneous extractions. The higher concentration of glucose produced from conditions n°1 and n°3 suggests that higher microwave power and longer extraction time can concur to increase the amount of hemicellulose in the extract. Hemicellulose extraction probably requires more extensive and harsher conditions compared to pectic polysaccharides, which may explain the lower proportions of xylose/mannose under conditions of treatment n°2.

The ratios of neutral monosaccharides and galacturonic acid can provide an important insight to the structural characteristics of rhamnogalacturonan I (Table 2.8). Optimal extraction conditions n°1, n°2, and n°3 produced ratios of GalA/Rha of 15.8/1, 10.8/1, and 35.5/1. In particular, the GalA/Rha ratio indicates that conditions n°1 and n°3 generated fewer homogalacturonan regions and more rhamnogalacturonan regions than did condition n°2. Similar results to those of conditions n°1 and n°3 were obtained previously from potato cell wall material (12/1) (Khodaei & Karboune, 2013), while condition n°2 generated GalA/Rha similar to that of pectin extracted from passion fruit peel (20-32:1). However, under all conditions, this ratio was by far lower than that of pectin extracted from banana peels (210-402:1) (Happi Emaga, Ronkart, Robert, Wathelet, & Paquot, 2008; Seixas et al., 2014). Gal/Ara ratios were all close to 1/1; treatments n°1 and n°3 had slightly lower Gal/Ara ratios, at 0.8/1 and 0.7/1, indicating that arabinan side chains are slightly higher in cranberry pectic polysaccharides compared to galactan chains. The Gal/Rha ratios ranged from 3.1/1-5.8/1 for the three optimal extraction conditions, which is lower compared to that of potato cell wall material (8.9/1), indicating that pectic polysaccharides isolated from cranberry pomace consist of shorter length galactan side chains, as was reported earlier by Khodaei and Karboune (2013) (Khodaei & Karboune, 2013). However, arabinan side chains were highly branched for conditions n°1 and n°3, given the ratios of Ara/Rha of 5.7/1 and 8/1, respectively.

Table 2.8. Optimal conditions generated using response surface methodology and experimental results, including monosaccharide profile and monosaccharide ratios.

Optimal Condition	**n°1**			**n°2**			**n°3**		
Alkaline concentration (M)	1.51			1.51			1.11		
Power (W/g)	65			35.33			65		
Pomace concentration (mg/ml)	16.33			38.67			38.67		
Time (min)	4.73			2.25			4.75		
		Predicted 95%			**Predicted 95%**			**Predicted 95%**	
Response [a]	**Experimental**	**Low**	**High**	**Experimental**	**Low**	**High**	**Experimental**	**Low**	**High**
Yield of Extract	28.65 (1.16)	21.20	29.90	17.90 (0.85)	15.55	24.00	14.97 (0.97)	14.21	22.57
Proportion of Total Phenolic Compounds	16.43 (1.86)	4.67	17.32	18.85 (1.81)	5.66	15.40	14.40 (4.79)	25.20	34.96
Proportion of Total Carbohydrates	83.57	82.18	96.24	81.15	82.68	93.22	85.60 (8.61)	62.81	74.58
Recovery of Total Carbohydrates	10.56 (0.90)	9.56	14.76	9.58 (0.97)	9.49	14.50	7.71 (0.67)	7.17	12.1
Recovery of Total Phenolic Compounds	57.00 (4.55)	38.72	100.0	61.12 (1.96)	21.14	77.36	30.86 (1.85)	57.1	100.0
Monosaccharide (%)	**Experimental**			**Experimental**			**Experimental**		
Rhamnose	2.41 (0.01)			4.50 (0.03)			1.48 (0.01)		
Arabinose	13.85 (0.02)			11.61 (0.05)			11.89 (0.05)		
Galactose	11.07 (0.03)			13.78 (0.06)			8.60 (0.05)		
Glucose	15.02 (0.05)			10.41 (0.07)			13.26 (0.07)		
Xylose/Mannose	19.56 (0.03)			11.10 (0.07)			17.19 (0.10)		
Uronic Acid	38.10 (1.97)			48.60 (3.37)			47.59 (3.35)		
Neutral Sugars	61.90 (1.58)			51.40 (2.80)			52.41 (2.49)		
Gal A/Rha	15.8			10.8			35.5		
Gal/Rha	4.6			3.1			5.8		
Ara/Rha	5.7			2.6			8.0		
Gal/Ara	0.8			1.2			0.7		

[a] Responses determined as % w/w.

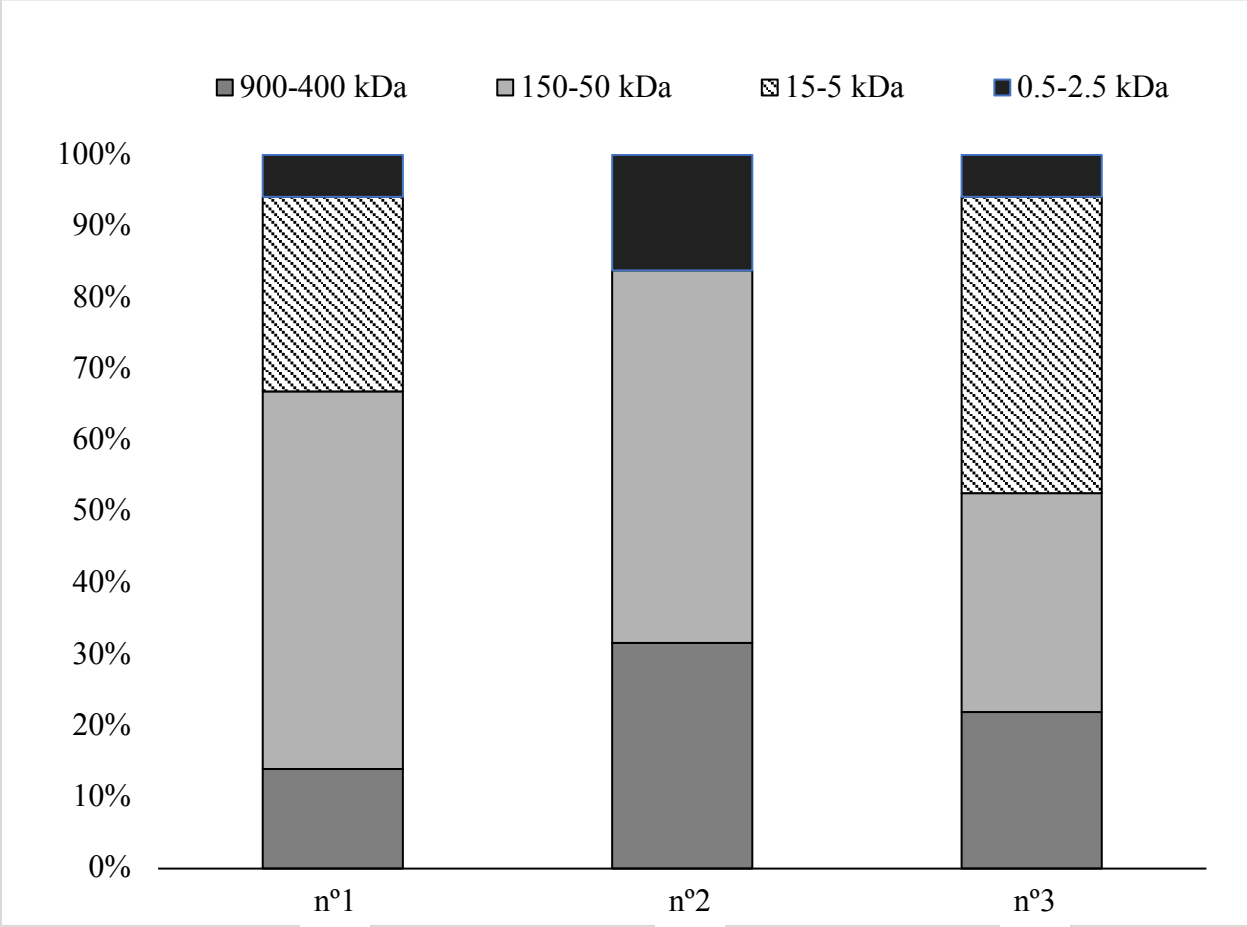

Figure 2.4. Molecular weight distribution of carbohydrates generated under top three optimal microwave-assisted extraction conditions determined using size exclusion chromatography.

2.5 Conclusion

This work demonstrated that microwave-assisted extraction proved to be a successful approach for production of an extract rich in cell wall components from cranberry pomace. All extraction conditions (acidic, alkaline, and sequential acidic-alkaline) produced extracts with varying composition. The sequential extraction was chosen as the preferred method of extraction, as opposed to singular extraction with either acid or base, due to opportunity to extract the maximum amount of material from cranberry pomace. Hence, this extraction method was subjected to optimization using Design Expert® to generate optimal extraction conditions. Optimal microwave extraction parameters for the production of an extract rich in phenolic compounds as well as large molecular weight cell wall polysaccharides was developed. The extracts generated were rich in both neutral sugars and uronic acids, and were high molecular weight, indicating the preservation of certain polysaccharides. The optimal extraction conditions were determined as 1.51 M NaOH, 65 W/g, 16.33 mg pomace/ml, and 4.73 min, generating an extract yield of 28.65% with a total phenolic and carbohydrate recovery of 57.0 % and 10.56%, respectively. The pectic polysaccharides generated were shown to be rich in rhamnogalacturonan regions (Gal A: Rha of 15.8:1), which are predominantly branched with arabinan chains (Ara: Rha of 5.7:1).

CONNECTING STATEMENT II

The efficiency of selected microwave-assisted extraction methods was investigated for the production of an extract rich in cell wall polysaccharides and polyphenolic compounds from cranberry pomace. The sequential acidic-alkaline microwave-assisted extraction being the most efficient one was optimized in chapter II, and the resulted optimized extracts were structurally characterized.

Chapter III describes the *in-vitro* TIM-1 digestion of the cranberry extract generated using the optimal conditions. This digestion was assessed by determining the changes in the molecular weight distribution, the total phenolic content, and in the monosaccharide profiles. In addition, the cranberry extract, its enzymatically hydrolyzed cranberry extract and the original cranberry pomace, as well as the oligofructose were subjected to *in-vitro* colonic fermentation using human fecal bacteria. This study is expected to determine the influence of the above selected products on the bacterial populations and the capability of the gut bacteria to use these products as fermentation substrates.

The results from chapter III will be submitted to a scientific journal, Food Chemistry.

Davis, E., Fliss, I., Karboune, S. (2018). *In-vitro* digestion and fermentation of cranberry extracts rich in cell wall polysaccharides and phenolic compounds (To be submitted).

CHAPTER III.

IN-VITRO DIGESTION AND FERMENTATION OF CRANBERRY EXTRACTS RICH IN CELL WALL POLYSACCHARIDES AND PHENOLIC COMPOUNDS

3.1. Abstract

Extracts produced from microwave-assisted, sequential acid-base extraction of cranberry pomace were subjected to in-vitro upper gastrointestinal digestion and colonic fermentation using a human fecal inoculum. The pectic polysaccharide regions of the original cranberry extract resisted gastrointestinal digestion, while the hemicellulosic polysaccharides were hydrolyzed to oligomers. A large proportion of neutral sugars (54.29%) were found in the jejunum dialysate while uronic acids were better recovered in the effluents (51.98%). The total phenolic content increased (227.2% increase compared to the total phenolic content of the meal) after gastrointestinal digestion, suggesting a liberation of phenolic compounds during digestion that are esterified to the polysaccharide matrix. The fermentation of the original cranberry extract, the enzyme-hydrolyzed cranberry extract, alcohol-insoluble cranberry pomace, and inulin (Orafti® P95) was also investigated. All cranberry products improved the population of the *Clostridia* cluster XIVa (Δlog bacteria = 1.3-2.5), important butyrate-producing gut microorganisms that improve the Firmicutes: Bacteroides ratio. The enzyme-hydrolyzed cranberry pomace and inulin increased the population of *Bifidobacterium* spp (Δlog = 0.5, 0.6, respectively), also contributors to butyrate production. Short chain fatty acid analysis revealed the production of large amounts of acetate, propionate, and butyrate for all substrates, with the total short chain fatty acids reaching 161.75 mmol/L for inulin, 159.67 mmol/L for the enzyme hydrolysis cranberry extract, 158.6 mmol/L for cranberry extract, and 145.49 mmol/L for cranberry pomace.

3.2. Introduction

The lower gastro-intestinal tract of human beings is a densely-populated region, rich in a diverse flora of microorganisms responsible for maintaining the gut health. Indeed, the gut microbiome plays an important role in many functions, including maintaining host immune function, inhibiting pathogen colonization, stimulating the vitamin production, producing short chain fatty acids and promoting healthy bowel functions (Bull & Plummer, 2014; M. Kumar et al., 2016; Thursby & Juge, 2017). The modulation of the gut microbiome can be achieved through the consumption of prebiotic ingredients being fermentable and non-digestible carbohydrates (Ríos-Covián et al., 2016). Health-promoting properties of prebiotics are dependent on their structural properties, including monosaccharide composition, degree of polymerization and the level of branching (Cardona et al., 2013; Louis et al., 2014; H.-D. Yoo et al., 2012). Different sources of non-digestible carbohydrates resulted in different growth patterns of colonic bacteria and metabolite production, in particular short chain fatty acid (SCFA) production (Ríos-Covián et al., 2016; Tabernero and Gómez de Cedrón, 2017). This leads to the selected modulation of the gut microbiome.

Among the non-digestible carbohydrates, inulin and fructooligosaccharides have been widely studied for their ability to stimulate the growth of *Bifidobacteria* spp. and to promote the production of the butyrate; however, evidence is emerging that the responses are dependent on the substrate composition and the size as well as the host itself (Rossi et al., 2005; Ramirez-Farias et al., 2009; Healey et al., 2017). Plant cell wall polysaccharides from food by-products have also emerged as attractive sources for the production of novel prebiotics, including uronic acid-rich and neutral sugar-rich non-digestible carbohydrates (Gómez et al., 2016, 2015; Gullón, Gullón, Sanz, Alonso, & Parajó, 2011; Olano-Martin, Gibson, & Rastall, 2002; Rose, Patterson, & Hamaker, 2010; van Laere, Hartemink, Bosveld, Schols, & Voragen, 2000). Most pectin-rich by-products, such as sugar beet pulp, citrus pulp, bergamot peel and apple pomace, have been investigated as sources of uronic acid-rich non-digestible carbohydrates, originating from homogalacturonan region of the cell wall (Aguirre, Jonkers, Troost, Roeselers, & Venema, 2014; Gómez, Yáñez, Parajó, & Alonso, 2014; Gulfi, Arrigoni, & Amadò, 2006; Mandalari et al., 2006). In addition, homogalacturonan-rich pectins have been shown to favour the production of large amounts of acetate as compared to the production of the butyrate upon the fermentation of

fructooligosaccharides (Gómez et al., 2014; Min et al., 2015; Cantu-Jungles et al., 2017a). However, large molecular weight neutral sugar-rich non-digestible carbohydrates show slower fermentation rates compared to smaller molecular weight oligo- and polysaccharides, suggesting that these larger, neutral polysaccharides will better reach the distal colon as fermentation substrates for inhabitant bacteria (Crittenden et al., 2002; Rycroft, Jones, Gibson, & Rastall, 2001). In addition, the gut microbiome responds differently to the neutral sugar-rich non-digestible carbohydrates depending on their plant origins. For instance, wheat arabinoxylan is degraded by a first utilization of xylose regions, followed by fermentation of arabinose chains, while soy and rice arabinoxylans are simply de-branched by intestinal bacterial (Rose et al., 2010). Arabinogalacto-, arabino-, rhamnogalacturono-, and arabinoxylooligosaccharides generated from soy, sugar beet pulp, apple and wheat polysaccharides are all fermented differently by colonic bacteria. The different fermentation patterns and selectivity of the fecal microbiota indicate that multiple sources of fermentable carbohydrates are necessary to encourage the growth and development as well as maintenance of a symbiotic microflora.

While carbohydrates constitute the major component of the plant cell wall, phenolic compounds are also present in the esterified form and tightly bound the cell wall polysaccharide network (Celep et al., 2014). Recent studies have demonstrated that the gastro-intestinal digestion can help liberate esterified phenolic compounds, making them bioavailable, and that some microorganisms found in the human gut are capable of utilizing phenolic compounds as source of energy (Correa-Betanzo et al., 2014; Jandhyala et al., 2015; Mosele et al., 2015). The production of an added value cell wall carbohydrates esterified to phenolic compounds could potentially enhance the delivery of these phenolic compounds to the lower gastrointestinal tract, where most of the diseases are originated (Jandhyala et al., 2015; Van den Abbeele et al., 2013).

Most of the studies investigated the "prebiotic potential" by assessing the fermentability by batch-cultured, individual or selectively mixed bacterial strains as opposed to the use of a complex ecosystem of multiple bacterial species that inhabit the human gut (Kurdi & Hansawasdi, 2015; Moreno-Vilet et al., 2014; Wongputtisin & Khanongnuch, 2015). While these methods provide some insight to fermentation patterns of individual bacterial strains and/or the mixed culture that was selected, they fail to take into account the symbiotic nature of the intestinal microflora and the importance of multiple metabolic pathways that lead to the proliferation of bacteria in the colon.

Orange and lemon peel waste, sugar beet pulp, apple pulp, and potato pulp are some materials that have been used to generate non-starch poly- and oligosaccharides that have been subjected to *in-vitro* fermentation using human fecal inoculum (Gómez, Gullón, et al., 2014; Gómez et al., 2016; Gullón et al., 2011; Khodaei, Fernandez, et al., 2016). These studies help to approximate the potential effects of non-digestible carbohydrates *in-vivo*. As far as the authors are aware, cranberry pomace has not been investigated as a potential source of fermentable carbohydrates. In the present study, the cranberry extract and its enzymatic hydrolysate, enriched with phenolic compounds and non-digestible carbohydrates, were produced by the sequential acidic/alkaline microwave-assisted treatment and the enzymatic treatment by Viscozyme, respectively. The *in vitro* digestibility of the cranberry polysaccharide extract and of its corresponding enzymatic hydrolysate were investigated using TIM-1 model in order to assess their ability to reach the colon intact. The total phenolic content, total carbohydrate content and monosaccharide profile, as well as the carbohydrate molecular weight distribution were assessed at each level of the gut system. Moreover, the continuous *in-vitro* fecal fermentation of cranberry pomace, cranberry polysaccharide extract, its enzymatic hydrolysate were assessed by evaluating shifts in faecal microbiota populations and short-chain fatty acid production. As an overall, this study is expected to contribute to the understandings of the prebiotic properties of cranberry pomace and of its components (polysaccharides, phenolic compounds).

3.3. Materials and Methods

All chemicals were reagent grade or HPLC grade for chromatographic analysis. NaOH was purchased from Fisher Scientific (Waltham, MA). Anyhydrous ethanol was purchased from Les Alcools de Commerce (Boucherville, QC). D-galactose, D-mannose, L-rhamnose, L-arabinose, D-xylose, D-glucose, D-galacturonic acid, 85% m-hydroxybiphenyl, sulfuric acid (95-98%, A.C.S grade), hydrochloric acid (37%, A.C.S. grade), phenol, Folin & Ciocalteu's phenol reagent, trifluoroacetic acid (Reagent Plus), sodium tetraborate, sulfamic acid (99.3%), potassium sulfamate, gallic acid, and salts were purchased from Sigma Aldrich (St. Louis, MO). Kestose, Nystose, and GF4 were purchased from Wako Chemicals USA (Cedarlane Laboratories, Burlington, ON).

3.3.1 Cranberry Extract Preparation

Dried ground pomace was prepared as previously described Chapter II. To prepare the extract, the pomace was extracted according to the optimal extraction conditions determined using Design Expert® software. 6.5g of pomace was suspended in 400 ml of 0.1M HCl in a glass beaker, stirred, and then extracted at 65 W/g pomace for 4.75 min in a Panasonic Inverter® (Canada) microwave with a rotating plate inside. Pomace was then filtered from the supernatant using Whatman GF/C glass microfiber filters. Supernatant was cooled in ice, neutralized with 2 M NaOH, and transferred to a refrigerated holding vessel. The spent pomace was then re-subjected to extraction with 1.5 M NaOH; the recovered supernatant was neutralized with 12 M HCl. The recovered extracts were ultra-filtered using a Millipore Prep/Scale spiral wound ultrafiltration TFF-6 cartridge (EMD Millipore, ON, Canada) with a 5 kDa cut-off. To recover the polysaccharides, the retentate was subjected to the ethanol precipitation (50% ethanol, 12hrs, 4 °C). The recovered extract by centrifugation (8,500 rpm for 20 min) was lyophilized .

3.3.2 Carbohydrate composition

3.3.2.1 Total Carbohydrate Content

Total carbohydrate content was assessed using the phenol-sulfuric acid assay (Dubois *et al*.,1956). Samples were prepared by dissolving lyophilized extracts (1-5 mg/ml) in distilled water and mixed for 20 min. 200 µl phenol solution (5% in water) was added to 400 µl of the sample preparation. 1000 µl of concentrated H_2SO_4 was added to the mixtures and then incubated for 10 min at room temperature. After incubation, samples were read at 480 and 490nm using spectrophotometer. Results were expressed as grams of glucose equivalents per gram of extract.

3.3.2.2 Total Uronic Sugars Content

The content of total uronic sugars was determined according to the assay of Blumenkrantz and Absoe-Hansen (1974). Samples were dissolved in distilled water (0.25-5 mg/ml) and vortexed for 20 minutes. 400 µl of the sample solution was added to 40 µl 4 M sulfamic acid-potassium sulfamate solution. After mixing, 2400 µl of 75 mM sodium tetraborate solution was added to the mixture. The mixtures were incubated for 20 min at 100 °C in water, after which they were cooled to room temperature. 80 µl of 0.15% m-hydroxyphenyl reagent was then added. After 10 min incubation, the absorbance of the mixtures were read at 525 nm and the results were expressed as grams of galacturonic acid equivalents per gram of extract.

3.3.2.3 Analysis of Monosaccharides Profile.

To determine the monosaccharides profile, acid hydrolysis was performed according to the method of Khodaei & Karboune (2013). 10 mg of sample was suspended in 200 µl of distilled water. An HCl/methanol mixture (1:4 v/v) was added to the reaction mixture to produce a reaction mixture of 1:5 (v/v). The mixtures were incubated at 70 °C for 24 hrs and thereafter boiled. 3000 µl of water were added to the hydrolyzed mixtures along with trifluoroacetic acid (TFA) in a ratio of 1:8 (v/v). After boiling at 100 °C to evaporate TFA (to a volume of 4000 µl), samples were neutralized with NaOH and freeze dried. A Dionex ICS 3000, equipped with pulsed amperometric detector and Carbopac PA-20 column, was used to analyze the total monosaccharide composition of acid hydrolyzed samples. The monosaccharides were eluted with a 5 mM sodium hydroxide at 0.4 ml/min flow rate. Neutral monosaccharides used to construct calibration curves include rhamnose, arabinose, galactose, glucose, xylose, and mannose.

3.3.3 Determination of total polyphenolic content

Total phenolic content of the extracts was performed using Folin-Ciocalteu's phenol assay. A 20 µl sample was added to 1580 µl distilled water along with 100 µl Folin's reagent. 300 µl of sodium carbonate solution was then added (250 g/L). The samples were then incubated at 40 °C for 30 min. Absorbance was read immediately at 765 nm. The results were expressed as gallic acid equivalents (GAE) per gram of extract.

3.3.4 Molecular weight distribution

The TSK-Gel 5000 PWXL and TSK-Gel 3000 PWXL (Tosoh Bioscience, Tokyo, Japan) were used in series to analyze the molecular weight distribution of the carbohydrates generated from the extraction and upon the TIM-1 digestion. A 0.1 M NaCl mobile phase was applied at 0.4 ml/min flow rate using 1525 Waters® Binary HPLC Pump (Milford, MA), and the carbohydrates were detected by a refractive index detector (Milford, MA). Dextran standards (Sigma Aldrich, St. Louis, MO) of varying molecular weights were used to generate calibration curves (50,150,270,140, 670 kDa).

3.3.5 Process scale up

The scale up of the sequential acidic/alkaline microwave-assisted extraction was carried out in order to produce higher amount of cranberry polysaccharide extract. A Panasonic Inverter® home microwave was calibrated using dH_2O (400 ml) to develop a correlation between the power level and the actual power (W) delivered to the solvent. The pomace was first suspended in 0.1M HCl

at a ratio of 0.016:1 (w/v) to yield a total volume of 4 L; the recovered pomace was then subjected to the extraction by 1.5M NaOH for 4.73 min at 65 W/g. The Recovered extracts were filtered using 90 mm Whatman® GF/C glass microfiber filters (fluffy filters prevented clogging) and then neutralized with either concentrated HCl or NaOH. Filtrates were pooled and ultrafiltrated. Ultrafiltration was carried out using a Millipore Prep/Scale spiral wound ultrafiltration TFF-6 cartridge (EMD Millipore, ON) with a 5 kDa cutoff to isolate larger molecular weight compounds. An easy-load Masterflex® pump (Cole Parmer, QC) was used to feed supernatant through the cartridge using high pressure. The filtrate was discarded (<5 kDa), while the retentate (>5 kDa) was ethanol-precipitated with 1 volume of anhydrous ethanol (final precipitation concentration of 50%). The extracts were left overnight at 4 °C to precipitate alcohol insoluble material, which was then centrifuged at 8500 rpm for 20 min. The recovered extracts were freeze-dried.

3.3.5.1 Enzymatic hydrolysis

The enzymatically hydrolyzed extract was produced using Viscozyme L® as a biocatalyst (Novozymes, Denmark). The Viscozyme L® multi-enzymatic preparation expresses pectinase (2996 U/ml), arabinanase (200 U/ml), galactanase (2636 U/ml), and rhamnogalacturonase (218 U/ml) activities (Khodaei, 2015). Preliminary trials demonstrated that 39.9 U of arabinose activity of Viscozyme L®/ml reaction media at 35 °C and 250 rpm for 2 hr was optimal for the generation of the extract enriched with oligosaccharides and oligomers. To stop the hydrolytic reaction, the reaction mixture was incubated for 5 min at 100 °C. Enzymatically hydrolyzed extracts were analysed using high performance liquid chromatography (HPLC)-size exclusion chromatography to ensure a minimal generation of mono- and disaccharides.

3.3.6 Evaluation of digestibility of cranberry polysaccharide/polyphenol extract

TIM-1 system (TNO, Nutrition and Food Research Institute, Zeist, Ne.) was used to assess the digestibility of the cranberry extract under intestinal conditions. The systems consists of four compartments simulating stomach, duodenum, jejunum and ileum working under controlled temperature of 37 °C. The cranberry polysaccharides extract was comprised of 7.81 g extract dissolved in 330 ml of the mixture of 1 M citrate buffer and small intestine electrolyte solution (SIES) at a ratio of 1:1.7 (v/v). 17,250 U amylase was added to the cranberry polysaccharide extract and agitated for 1-2 min before being ‘fed’ to stomach. The stomach consisted of a total volume of 310 ml with an initial content of 10 ml of gastric juice (6000U pepsin and 375U lipase in gastric electrolyte solution composed of 6.2 g/L NaCl, 2.2 g/L KCl, 0.3 g/L $CaCl_2$ and 1.5 g/L

$NaHCO_3$). Gastric secretions included gastric juice at a pH 4 and at a flow rate of 0.25 ml/min with 600U/ml pepsin and 37.5U/ml lipase in gastric electrolyte solution. 300 ml of meal was added to the stomach at t=0. Initial pH in stomach was 5.8, which was dropped gradually to 1.7 and 1.5 by injecting 1 M hydrochloric acid, after 60 and 120 min of digestion, respectively, and remained constant at 1.5 from 180 to 300 min. The total volume of the duodenum was 55 ml at pH 6.3. Initial content of the duodenum included 15g of 21% pancreatin solution, 30g of 4% bile solution, 1 ml of 2 mg/ml trypsin solution, and 15g SIES (0.5 g/L NaCl, 0.6 g/L KCl, 0.3g/L $CaCl_2$, pH 7.0). Duodenum secretions included 21% pancreatin solution at 0.25 ml/min, 4% bile solution for t=0-30 min at 0.5 ml/min then 2% bile at 0.5 ml/min for t=30-300 min, and SIES or 1M $NaHCO_3$ at 0.25 ml/min. The total volume of the jejunum (pH 6.5) and ileum (pH 7.4) was 115 ml each. Initial content of the jejunum and ileum comprised SIES with secretions of 1M $NaCO_3$ if necessary. Dialysis fluid was SIES at 10 ml/min. Samplings of effluents, jejunal dialysates, ileal dialysates, and chymes were collected every 60 min for 300 min total digestion time; chymes were taken only at t=300 min. Enzymes in effluent portions from each sampling time were inactivated during a 5-minute incubation time at 75 °C immediately after sampling. Effluents and dialysates were freeze dried within 72 hrs for complete dryness and stored at room temperature until further analysis.

3.3.7 Fermentation of selected cranberry products

Immobilization of Fecal Inoculum. Fresh human feces were taken from a healthy donor who had not taken antibiotics for at least 3 months before. Fecal sample (25%, w/v) was suspended in peptone water (0.1 %, w/v) containing 0.05% (w/v) L-cysteine HCl at 37 °C. The fecal inoculum was recovered by centrifugation (700 rpm, 1 min). The fecal inoculum was entrapped into gum-based beads according to the dispersion method of Cinquin et *al*. (2004). In an anaerobic chamber, the fecal inoculum (2%, v/v) was added to sterilized gum solution, made of xanthan gum (0.25%, w/v) and gellan (2.5%, w/v), in trisodium citrate solution (0.2%, w/v) at 40 °C. 250 ml of the gum solution was poured slowly into 500 ml canola oil (hydrophobic media) to form beads. The recovered beads were suspended in autoclaved 0.1 M calcium chloride ($CaCl_2$) solution for 30 min under vigorous shaking in order to harden them. Beads were then washed with 1L wash solution (0.03 M $CaCl_2$, 0.27 M KCl) and were sieved to obtain beads with a diameter between 1-2 mm. 75-80 g of beads were placed in a 500 ml bioreactor (BIOSTAT Qplus, Sartorius AG, Goettingen, Germany) containing 250 ml of sterile Macfarlane media under anaerobic conditions. The entire process was performed in less than 2 hours.

Bioreactor Preparation and Fermentation. Macfarlane media was used and consisted of carbohydrate sources (2 g/L citrus pectin, 2 g/L arabinogalactan from larch tree, 2 g/L xylan from corn, 1 g/L guar gum, 1 g/L inulin from chicory, 5 g/L potato starch), nitrogen sources (4 g/L porcine mucin type II, 3 g/L casein hydrolysate, 5 g/L peptone, 5 g/L tryptone, 4.5 g/L yeast extract), bile salt n°3 (0.05 g/L), tween 80 (1 ml/L), and 5% w/v hemin solution (1 g/L), salts (0.5 g/L KH_2PO_4, 1.5 g/L $NaHCO_3$, 4.5 g/L NaCl, 4.5 g/L KCl, 1.25 g/L anhydrous $MgSO_4$, 0.1 g/L $CaCl_2$, 0.2 g/L $MnCl_2{\cdot}4H_2O$, $FeSO_4{\cdot}7H_2O$), 5% cysteine HCl (16 g/L) and a vitamin solution (0.5 g/L). Vitamin solution consisted of pyridoxine HCl (20 mg/L), 4-aminobenzoic acid (10 mg/L), nicotinic acid (10 mg/L), biotin (4 mg/L), folic acid (4 mg/L), cyanocobalamine (1 mg/L), thiamine (8 mg/L), riboflavin (10 mg/L), menadione (2 mg/L), phyloquinone (0.005 mg/L), panthothenate (20 mg/L). Anaerobic conditions were achieved using a continuous flow of oxygen free anaerobic gas mixture in the bioreactors and oxygen free nitrogen gas bubbling into the Macfarlane media (Praxair, QC). Fermentation was carried at 37 °C, and the pH was maintained at 6.2 with 5 M NaOH throughout the duration of fermentation. After the 3-day period of fermentation, continuous fermentation was started and monitored until bacterial populations were stable, approximately 16 days. Indeed, the inoculum reactor was used to inoculate continuously two test reactors throughout the duration of the fermentation. 100% Macfarlane media was always used in the inoculum reactor. After 16 day stabilization period, the fermentation of a negative control consisting of 25% of the original carbohydrate content was first carried out. Then after, 75% of carbohydrate source of culture medium were substituted with the original cranberry pomace, the cranberry extract, its corresponding enzymatic hydrolysate and inulin as a standard reference (Orafti® P95, 1.2 kDa). Each test included 72h stabilization period, followed by a 72h fermentation (Figure 3.1). After each test period, the two test reactors were washed with concentrated sodium hypochlorite solution (10% w/v) and with sterile distilled water multiple times until the pH returned to 6.8-7.2.

Quantification and Identification of Bacteria. Sampling was performed in a sterile fashion every 24 hours in all reactors. 100 uL of media was suspended in 900 ul of 0.1% peptone water reduced with L-cysteine to yield a final concentration of 0.05% (pH was adjusted to 6.2-6.8 with 1M NaOH). 2.5 µL of propionum monoazide (PMA) solution (20 mM in 20% dimethyl-sulfoxide) was added and mixed well, then incubated in the dark for 5 min. Samples were then incubated laying upon ice under a halogen light for 5 min.

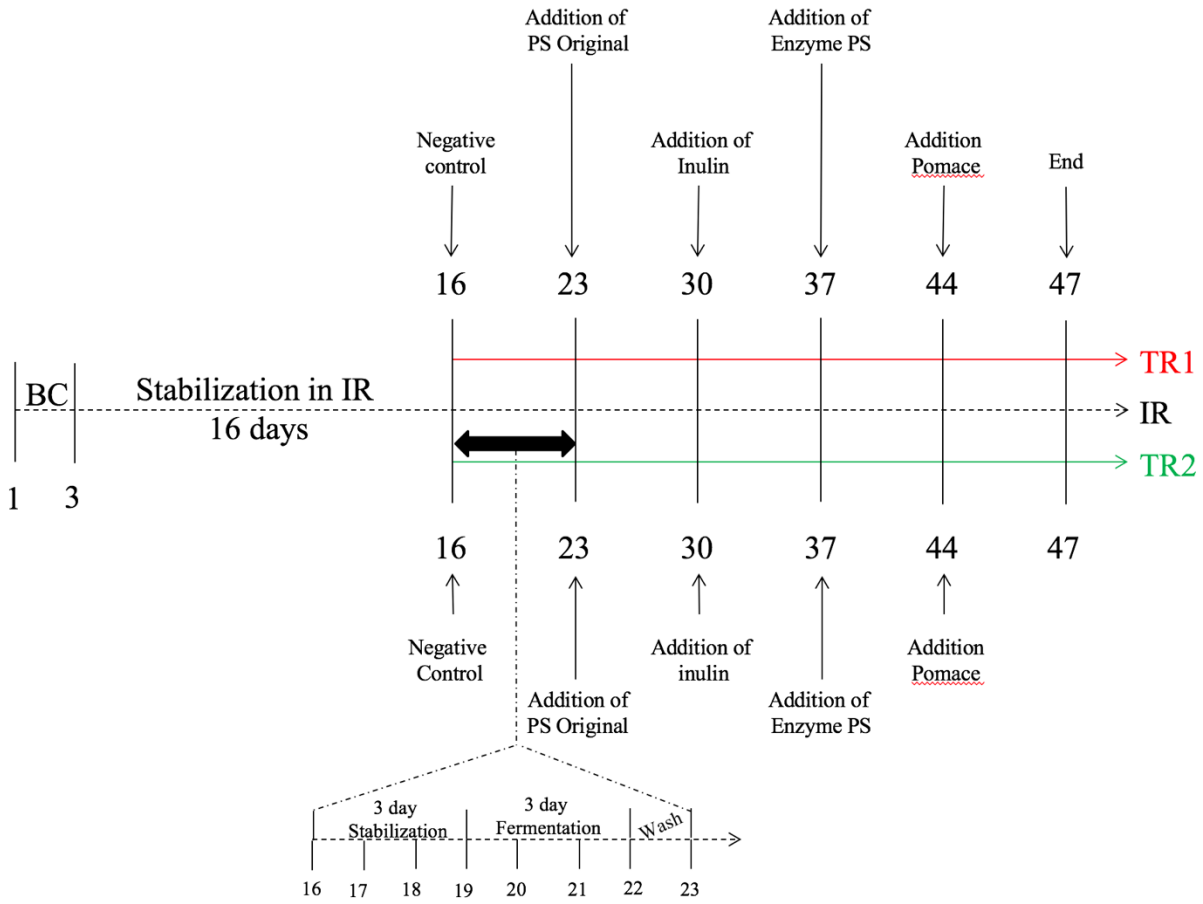

Figure 3.1. Schematic representation of fermentation timeline in days (numbered). Days may have varied slightly due to washing times. BC: bead colonization period, IR: inoculum reactor, TR: test reactor, PS Original: cranberry extract/polysaccharide.

Samples were then centrifuged at 14,500 rpm at 4 °C and the supernatant was discarded. Pellet was frozen at -80 °C until DNA extraction. An enzymatic lysis step was carried out before DNA extraction using a DNeasy PowerSoil kit (Qiagen, ON, Canada). The enzymatic lysis consisted of washing PMA treated samples twice with 700 µL tris-EDTA buffer (10 mM tris-HCl, 1 mM EDTA) and centrifuging at 10.4 ×g for 5 minutes. The pellet was then suspended in 200 uL of the above tris-EDTA buffer containing 40U mutanolysin, 5 µg/ml proteinase K, and 40 mg/ml lysozyme and incubated for 1 hour at 37 °C. Samples were centrifuged at 10.4 ×g for 3 minutes and the pellet was used for DNA extraction with the above-mentioned kit. Identification and quantification of bacterial strains was performed using real-time polymerase chain reaction (qPCR) and droplet-digital polymerase chain reaction (ddPCR). Primers used for bacterial identification are listed below in Table 3.1. Standard curves were constructed for each bacterial group using dd-PCR, where 10 µL Evagreen® supermix, 1 µL forward primer, 1 µL reverse primer, 4 uL cDNA, and 4 uL ultra-pure, DNase-free water were added to each 20 uL well and droplets were formed using a QX200 droplet generator (Bio-Rad, Mississauga, ON) and PCR was performed using a T100 thermal cycler (Bio-Rad, Mississauga, ON). Droplets were then quantified in a QX200 droplet reader (Bio-Rad, Mississauga, ON). DNA samples from fermentation were identified and quantified using qPCR. Extracted DNA was diluted 10-fold and used in the following qPCR reaction mixture: 12.5 µL fast SYBR green, 1 µL forward primer, 1 µL reverse primer, 5.5 µL ultra-pure, DNase-free water, and 5 µL 10x diluted DNA. An ABI 7500 fast real-time PCR system was used to analyze 96-well plates (Applied Biosystems, ON, Canada).

3.3.8 Organic Acid Analysis

Culture samples recovered at the end of the 16 day-stabilization period and of each fermentation period were centrifuged (10,000 ×g, 10 min, 4 °C) and analyzed for their organic acid contents using a HPLC system (Waters, Milford, MA, USA) equipped with an ICSep-Ion-300 (300 x 7.8 mm) column (Transgenomics, San Jose, CA, USA) and a differential refractometer detector (Model 2142, LKB, Bromma, Sweden). A mobile phase composed of 8.5 mM H_2SO_4 at 0.4 ml/min flow rate was applied.

Table 3.1. Primers used for bacterial identification and quantification during qPCR and ddPCR analysis.

Group	Gene	Name	Sequence 5'-3'	Amplicon Size (pb)	References
Total Flora	16S	Uni 331F	TCCTACGGGAGGCAGCAGT	466	(Nadkarni, Martin, Jacques, & Hunter, 2002)
		Uni 797R	GGACTACCAGGGTATCTAATCCTGTT		
Clostridial cluster IV	16S	Clep866mF	TTAACACAATAAGTWATCCACCTGG	314	(Ramirez-Farias et al., 2009)
		Clept1240mR	ACCTTCCTCCGTTTTGTCAAC		
Roseburia spp.* and E. *rectale	16S	RrecF	GCGGTRCGGCAAGTCTGA	81	(Ramirez-Farias et al., 2009)
		Rrec630mR	CCTCCGACACTCTAGTMCGAC		
***Bacteroides - Prevotella* group**	16S	F	GGTGTCGGCTTAAGTGCCAT	140	(Rinttilä, Kassinen, Malinen, Krogius, & Palva, 2004)
		R	CGGAYGTAAGGGCCGTGC		
Lactobacillus/ Leuconostoc/ Pediococcus	16S	F_Lacto 05	AGCAGTAGGGAATCTTCCA	350	(Furet et al., 2009)
		R_Lacto 04	CGCCACTGGTGTTCYTCCATATA		
Enterobacteriaceae	16S	Eco1457F	CATTGACGTTACCCGCAGAAGAAGC	195	(Bartosch, Fite, Macfarlane, & Mcmurdo, 2004)
		Eco1652R	CTCTACGAGACTCAAGCTTGC		
Bifidobacterium spp.	16S	F	TCGCGTCYGGTGTGAAAG	243	(Rinttilä et al., 2004)
		R	CCACATCCAGCRTCCAC		
Enterococcus spp.	16S	F	CCCTTATTGTTAGTTGCCATCATT	144	(Rinttilä et al., 2004)
		R	ACTCGTTGTACTTCCCATTGT		

3.4 Results and Discussion

3.4.1. Effect of the digestion on the carbohydrate profile and total phenolic content of the cranberry polysaccharide/polyphenol extract

The cranberry polysaccharide/polyphenol extract was isolated from cranberry pomace using a sequential acidic/basic microwave-assisted extraction. The optimal extraction conditions correspond to 16.33 mg pomace/ml media, 65 W/g pomace, 1.5M NaOH, and extraction time of 4.73 min. The recovered extract (meal) was composed of 2.0% of oligosaccharides (DP of 2-10), 0 % oligomers (DP of 10-20) and 98.0% of polysaccharides (Table 3.4). In order to determine the colonic persistence, the digestibility of cranberry polysaccharide extract was assessed using the TIM-1 model. The carbohydrate composition, the polyphenolic content, the monosaccharide profile and the molecular weight distribution of the initial fed (stomach) and digesta are reported in Tables 3.2, 3.3 and 3.4. The results (Table 3.2) shows that the proportion of uronic acids, neutral sugars, and phenolic compounds of the meal (initial fed) are under-estimated as compared to the digesta. This can be most likely attributed to the complex structure of the polysaccharide and the polyphenolic compounds network that may have resisted to the acid hydrolysis carried out prior the quantification. The results also indicate that uronic acid-based carbohydrates resisted to digestion as 51.98% and 22.29% of uronic acids were detected in the effluents and chymes, respectively. Only 18.79% and 6.94% of uronic acids were absorbed via the jejunum and ileum, respectively. It can be hypothesized that the uronic acid-based carbohydrates of cranberry extract exhibit a high colonic persistence to digestion and could potentially act as prebiotic materials. However, neutral sugar regions of the oligo- and polysaccharides were hydrolyzed more extensively. Indeed, 75% of total neutral sugars were detected in the jejunum and ileum dialysates, at a proportion of 54.29% and 20.77%, respectively. Only 15.05 and 9.89 % of neutral sugars were detected in the effluents and the chymes, respectively. These results confirm that neutral sugar regions of polysaccharides were much less resistant to digestion than uronic acid regions. The hydrolysis of the neutral sugar regions is most likely due to the acidic gastric digestion, given the low pH of the stomach compartment; while the homogalacturonan regions of pectin are generally preserved under acidic conditions (Khodaei & Karboune, 2013).

The polyphenolic content of the cranberry extract was enhanced upon digestion. Indeed, the total polyphenolic content recovered in all digesta was 2.3 times higher (Table 3.2) than that of the

initial fed meal. This result reveals the high bioavailability of polyphenolic compounds upon gastric digestion by de-esterifying the phenolic compounds and affecting their binding affinity to the plant polysaccharides (Gullon et al., 2015). Hydrolysis of polyphenolic compounds in oligomer or polymer forms may have occurred as 106.63 mg and 31.15 mg GAE/g extract were recovered in the jejunal and the ileal dialysates, respectively. This is due to low gastric pH and salivary enzymes having a hydrolytic effect on phenolic compounds. Campos-Vega *et al.* (2015) reported that phenolic compounds from spent coffee were reduced by 38% upon salivary digestion. According to the same study, it was also found that gastric conditions lead to an increase in total phenolic content, due to the hydrolysis of condensed tannins and flavanoids (Campos-Vega et al., 2015). Furthermore, a study investigating blueberry polyphenols demonstrated remarkable stability after gastric digestion, with 94% recovery. However, blueberry anthocyanins underwent structural changes via benzopyran ring cleavage after the change between acidic to alkaline environment, leading to the generation of monomeric phenolic compounds (Correa-Betanzo et al., 2014). Similar results were reported upon the digestion of pomegranate extracts, where the concentration of ellagic acid increased after digestion, due to the hydrolysis of polyphenolic compounds from the food matrix (Mosele et al., 2015). The authors also hypothesize that phenolic compounds are so firmly bound to the food matrix in the undigested material, making them inaccessible for quantification.

The results (Table 3.2) also show that certain polyphenolic compounds resisted to the digestion and the acidic conditions of the stomach as demonstrated by the recovery of 134.97 (40.92% of total phenolic content of digesta) and 57.12 (17.31% of total phenolic content of digesta) mg GAE/ g extract, in the effluents and the chymes, respectively. This can be attributed to the fact that some of polyphenolic compounds remained esterified to cell wall polysaccharides, which exhibited a protective effect against gastric and enzymatic digestion. It could also be hypothesized that large molecular weight polyphenols underwent some degree of hydrolysis, but not sufficiently enough for them to be absorbed by the dialysis membranes (11 kDa cutoff) at the jejunal and ileal level. While these findings demonstrate that some phenolic compounds will reach the colon to act as a potential carbon source for bacteria, it also shows the importance of multicomponent food matrices and the protective effects that are exerted by one component to another.

Table 3.2. Total phenolic content as well as total amounts and proportions of uronic and neutral sugars in meal and digestas from TIM-1 gastrointestinal digestion.

	Uronic Acids (g)		Neutral Sugars (g)		% of Uronic Acids[c]	% of Neutral Sugars[d]	% of Total CHO[e]	% Uronic Acids of CHO[f]	%Neutral Sugars of CHO[f]	Total Phenolics[g]	
Meal	1.62	(0.02)[a]	0.70	(0.16)	69.44[b]	29.89[b]	-	-	-	145.18	(28.62)
Effluents	0.63	(0.08)	0.65	(0.10)	51.98	15.05	24.53	11.41	11.75	134.97	(20.87)
Chymes	0.27	(0.04)	0.43	(0.20)	22.29	9.89	13.32	4.89	7.72	57.12	(9.72)
Jejunum dialysate	0.23	(0.03)	2.35	(0.42)	18.79	54.29	47.08	4.12	42.37	106.63	(32.83)
Ileum dialysate	0.08	(0.02)	0.90	(0.21)	6.94	20.77	18.08	1.52	16.21	31.15	(12.54)
Total	1.217		4.327							329.87	

[a]Standard deviation in brackets.

[b]Proportion of total carbohydrates in meal only (meal uronic acids+ meal neutral sugars=total meal carbohydrates).

[c]The proportions of uronic acids of effluents, chymes, and dialysates as a function of the total uronic acids of the digesta.

[d]The proportions of neutral sugars of effluents, chymes, and dialysates as a function of the total neutral sugars of the digesta.

[e]The proportion of digesta carbohydrates (neutral+uronic) as a function of total carbohydrates of all digestas.

[f]Distribution of both neutral sugars and uronic acids as a function of total carbohydrates (uronic+neutral).

[g]Expressed as grams of gallic acid equivalents per gram of digesta.

3.4.2 Monosaccharide Composition and Molecular Weight Distribution of Digestas

Table 3.3 demonstrates the monosaccharide profile of the initial fed and the digesta in each compartment. The monosaccharide profile of the initial fed (stomach) shows that the contents of Rha, Ara, Gal, Glc+Xyl+Man and GalA in the polysaccharide/oligomer fraction were 0.07, 0.11, 0.14, and 0.38 g, respectively (Table 3.3) with 69.44% uronic acid and 29.89% neutral monosaccharides (Table 3.2). The monosaccharide profiles of effluents and chymes show that a significant proportion of the neutral sugars (50.75 and 61.21% of the recovered carbohydrates in the effluent and chyme, respectively) were resistant to digestion, in particular rhamnose, arabinose, galactose, and xylose; while significant amounts of glucose and galactose were found in the dialysates compared to the other neutral sugars. Rhamnose was recovered in both the effluents (0.03 g, 0.46% of total digesta) as well as in the chyme (0.01 g, 0.1% of total digesta; Table 3.3), but was not detected in the jejunum and ileum dialysates. This implies that the rhamnogalacturonan I region of the cranberry pectic oligo- and polysaccharides were resistant to digestion. Arabinose was also much more prevalent in the effluents and chymes compared to the dialysates. Indeed, some arabinose were absorbed by the jejunum (0.01 g) and ileum (0.01 g), but relatively small amounts (0.31% total digesta; Table 3.3) compared to what remained after digestion in the effluent (0.05 g) and chyme (0.02 g) as the contribution of arabinose in the effluent and chymes was 1.26% of the total digesta (Table 3.3). However, large amounts (28.57% of the total digesta) of galactose were found in the dialysates revealing their absorption in both the jejunum and ileum (Table 3.3); these results indicate the extensive hydrolysis of galactose-containing poly- or oligosaccharides into hydrolysates with less than 11 kDa (the cut-off of the absorption membranes). Along with the detection of arabinose in the dialysates, it can be hypothesized that the galactose may be originated from the arabinogalactan. Golovchenko *et al.* (2012) also suggested that acidic conditions of the stomach can hydrolyze galactan chains obtained from onions. As compared to galactose, glucose showed a similar trend, representing 29.07% of the total digesta that was absorbed by the jejununal and ileal dialysis membranes. This demonstrates that glucose-containing poly- or oligosaccharides were susceptible to hydrolysis during digestion. Mannose and xylose were recovered largely in the effluent and chyme rather than the dialysates. The undigested portions of mannose found in the effluent and chyme was 0.46% and 0.08% of the total digesta, respectively; and xylose was 1.38% and 0.39% of the total digesta, respectively (Table 3.3). The proportions of mannose and xylose in the dialysates represented 1.04% and 0.71% of the total digesta, respectively.

Table 3.3. Monosaccharide composition and their distribution in the respective compartments of the TIM-1 system, as well as their proportions as a function of the recovered total digestas.

	MEAL		EFFLUENTS			CHYMES			JEJUNUM DIALYSATE			ILEUM DIALYSATE		
	CHO (g)	**% of Meal[b]**	**CHO (g)**	**% of Effluents[c]**	**% total Digesta[d]**	**CHO (g)**	**% of Chymes[e]**	**% total Digesta**	**CHO (g)**	**% of Jejunum[f]**	**% total Digesta**	**CHO (g)**	**% of Ileum[g]**	**% total Digesta**
Rhamnose	0.07 (0.00)[a]	2.83	0.03 (0.00)	1.99	0.46	0.01 (0.00)	0.81	0.10	-	0.00	0.00	-	0.00	0.00
Arabinose	0.11 (0.03)	4.83	0.05 (0.01)	4.10	0.95	0.02 (0.00)	2.47	0.31	0.01 (0.00)	0.43	0.20	0.01 (0.00)	0.63	0.11
Galactose	0.14 (0.02)	6.07	0.23 (0.01)	17.82	4.13	0.18 (0.01)	25.32	3.19	1.10 (0.06)	42.46	20.12	0.46 (0.02)	46.76	8.45
Glucose	0.16 (0.03)	7.01	0.24 (0.04)	18.93	4.38	0.20 (0.05)	28.90	3.65	1.17 (0.21)	45.31	21.47	0.41 (0.01)	42.06	7.60
Mannose	0.07 (0.00)	2.94	0.03 (0.00)	1.97	0.46	0.01 (0.00)	0.66	0.08	0.04 (0.00)	1.66	0.79	0.01 (0.01)	1.38	0.25
Xylose	0.15 (0.00)	6.42	0.08 (0.00)	5.94	1.38	0.02 (0.00)	3.05	0.39	0.03 (0.01)	1.27	0.60	0.01 (0.00)	0.59	0.11
Uronic Sugars	1.62	69.91	0.63	49.27	11.41	0.27	38.79	4.89	0.23	8.87	4.20	0.08	8.58	1.55
Total Compartment (g)	2.32		1.28			0.70			2.58			0.98		
Total Digesta (g)	5.54													

[a] ± standard deviation in brackets.

[b] Distribution of monosaccharides and total uronic acids relative to total carbohydrates in meal.

[c] Distribution of monosaccharides and total uronic acids relative to total carbohydrates in all effluents (t=60 min to t=300 min).

[d] Distrubtion of monosaccharides and total uronic acids relative to the total carbohydrates of the sum of all digestas (t=60 min to t=300 min).

[e] Distribution of monosaccharides and total uronic acids relative to total carbohydrates in all chymes (t=60 min to t=300 min).

[f] Distribution of monosaccharides and total uronic acids relative to total carbohydrates in all jejunum dialysates (t=60 min to t=300 min).

[g] Distribution of monosaccharides and total uronic acids relative to total carbohydrates in all ileum dialysates (t=60 min to t=300 min).

[h] Sum of monosaccharides and uronic acids in each respective digestive compartment.

Given the occurrence of glucose, mannose, and xylose in the dialysates, we can infer that the hemicellulosic polysaccharides present in the cranberry extract underwent some extensive hydrolysis to reduce their molecular weight below 11 kDa. This may be explained due to the acidic conditions of the stomach (pH 1.5-2) that have a hydrolytic effect on the oligo- and polysaccharides composed of neutral sugars (Hilz et al., 2005; Hu et al., 2013).

Table 3.4 shows the molecular weight distribution after TIM-1 digestion of cranberry extract. The initial meal consisted of 2.0% of oligosaccharides and 98% of polysaccharides. More specifically, the molecular weight distribution of the cranberry polysaccharide/polyphenol extract meal was composed of 24% of 605.7 kDa, 49% of 122 kDa and 25% of 9.4 kDa polysaccharides with 2% 0.75 kDa oligosaccharides. 22% of the initial meal was recovered in the undigested material as polysaccharides (2.49%), oligomers (0.14%), and oligosaccharides (19.34%). Oligosaccharides were largely found in the jejunum dialysate (46.90%) and ileum dialysate (30.86%). The decrease of polysaccharides from the meal and the increase of oligosaccharides indicate the generation of smaller molecular weight carbohydrates under acidic conditions of the stomach that were absorbed by the jejunum and ileum. We speculate that the hemicellulosic polysaccharides underwent the most hydrolysis as shown by the monosaccharide profile (Table 3.3). Similarly, a neutral sugar-rich polysaccharide obtained from *Plantago asiatica* seeds underwent significant hydrolysis from 1903.1 kDa to 8.9 kDa after gastric digestion and further decreased to 4.7 kDa during intestinal digestion (Hu et al., 2013). It is possible that some oligosaccharides generated by the gastric digestion of cranberry extract meal may have been absorbed by the hollow-fiber membranes (11 kDa cut-off) and did not reach the effluents. Khodaei *et al.* (2016) performed TIM-1 digestion on potato rhamnogalacturonan and also reported that the hollow fiber membranes are may not be entirely representative of digestion, as small oligosaccharides are often broken down into monosaccharides before they are absorbed via the bloodstream. Given this, TIM-1 digestion may have underestimated the proportion of oligosaccharides that would reach the colon as fermentable substrates. However, the overall results reveal that the large polysaccharides, recovered in the effluents and chymes, were mainly pectic polysaccharides consisting of homogalacturonan and rhamnogalacturonan I. Similarly, it has also been reported that pectic polysaccharides from kiwi fruit, composed of homogalacturonan and rhamnogalacturonan, are resistant to digestion and remained relatively intact (Carnachan et al., 2012).

Table 3.4. Molecular weight distribution of digestas recovered formed from duplicate digestion of cranberry extract, determined using size exclusion chromatography.[a]

Digestion Experiment #1	%Oligosaccharides (DP 2-10)	%Oligomers (DP 10-20)	%Polysaccharides (DP>20)
Meal[b]	2.0	-	98.0
Effluent	8.38	0.14	1.22
Chymes	10.96	-	1.27
Jejunum dialysate	46.90	0.14	-
Ileum dialysate	30.86	0.13	-

[a] Proportions of oligosaccharides, oligomers, and polysaccharides in digestas are calculated relative to the total carbohydrates of sum of digestas.

[b] Proportions for meal are relative to total amount of carbohydrates in meal.

3.4.3 In-Vitro Fermentation of Cranberry Materials and Inulin

3.4.3.1 Population shifts in bacterial species

Cranberry extract as well as an enzymatically hydrolyzed cranberry extract were examined for their fermentability. The cranberry extract consisted of 24% of 605.7 kDa, 49% of 122 kDa and 25% of 9.4 kDa polysaccharides with 2% 0.75 kDa oligosaccharides. The high proportion of poly- in the cranberry extract will demonstrate the fermentability of large molecular weight carbohydrates isolated from cranberry pomace. The enzymatically hydrolyzed cranberry extract by Viscozyme L® consisted of 60% of 3.5 kDa oligosaccharides with 10% of 1.4 kDa and 10% 0.7 kDa oligosaccharides and 20% disaccharides. Viscozyme L® has been previously applied to plant cell wall material in many studies due to its cell wall hydrolysis capabilities (Anthon & Barrett, 2008; Baby & Ranganathan, 2016; N. Chen et al., 2015; De Camargo et al., 2016; Duenas et al., 2006). The cranberry pomace used as starting materials for the generation of the cranberry extract as well as oligofructose (Orafti® P95, 1.2 kDa, inulin-type) were also investigated for their fermentability by colonic bacteria.

The prebiotic properties of the cranberry pomace, the polysaccharides/polyphenols-enriched cranberry extract and its hydrolysates were assessed by measuring their abilities to support the growth of mixed bacterial populations from human faecal inoculum. Because prebiotic oligofructose is known for their ability to enhance the growth rate of *Bifidobacterium* and decrease that of *Clostridium,* it was used as a positive control (Gómez et al., 2016; Khodaei, Fernandez, et al., 2016). The populations of all bacteria were relatively high in the donor fecal matter and remained more or less with the same intensity after 16 days of stabilization (Table 3.5). Most notably, *Enterococcus* spp. population was well recovered after the 16-day stabilization period. *Lactobacillus* spp. was present in the feces; however, it did not grow well and was undetectable at stabilization day 16. It is important to note that the donor followed a long-term omnivorous diet, which is a diet rich in fermentable carbohydrates. Bacterial populations vary significantly between populations and individuals, suggesting that the interactions and symbiosis within the bacterial community are more important than the abundance of specific bacterial species (David et al., 2014; Healey et al., 2017). Regardless, it is still believed that a higher *Firmicutes:Bacteroidetes* is more beneficial for the host (Brahma et al., 2017; Jandhyala et al., 2015). As far as the authors are aware, this study is the first time to investigate the fermentability of cranberry materials.

Table 3.5. Bacteria counts of feces, the last day of stabilization, and during carbohydrate-modified Macfarlane media (log cells/ml). Change in bacterial count (Δlog cells/ml) is the difference between last day of stabilization period (data not shown) and last day (72h) of fermentation of carbohydrate-modified Macfarlane media.

	Experiment/Day							Change in Bacterial Count (Δlog)			
	Feces[b]	**Day 16**	**25% CHO[c]**	**CPS[d]**	**EPS[e]**	**Pomace[f]**	**Inulin**	**CPS**	**EPS**	**Pomace**	**Inulin**
All Bacteria	10.56 (0.79)[a]	10.47 (0.29)	9.96 (0.43)	10.12 (0.03)	10.27 (0.20)	10.12 (0.05)	10.40 (0.12)	0.2	0.1	-0.1	0.1
Bacteroides/Prevotella	10.36 (0.13)	9.95 (0.11)	9.75 (0.07)	9.91 (0.1)	10.15 (0.03)	9.93 (0.08)	10.31 (0.01)	0.1	-0.1	-0.2	0.1
***Enterococcus* spp.**	4.65 (0.3)	8.26 (0.15)	8.27 (0.15)	8.78 (0.85)	9.58 (0.04)	8.92 (0.06)	9.59 (0.08)	0.0	0.4	-0.2	0.4
Enterobacteriacae	7.54 (0.11)	8.19 (0.07)	7.61 (0.02)	8.61 (0.07)	8.81 (0.03)	9.35 (0.29)	8.70 (0.03)	0.0	0.1	1.5	0.5
***Bifidobacterium* spp.**	8.56 (0.30)	7.31 (0.11)	6.18 (0.26)	6.12 (0.22)	6.81 (0.05)	6.25 (0.22)	7.05 (0.13)	-0.1	0.6	0.2	0.5
***Clostridia* Cluster IV**	9.82 (0.16)	8.96 (0.07)	8.12 (0.17)	8.94 (0.16)	8.72 (0.30)	9.47 (0.09)	8.96 (0.19)	0.0	0.0	0.7	0.6
***Clostridia* Cluster XIVa**	10.81 (0.08)	8.17 (0.04)	8.53 (0.26)	8.64 (0.00)	8.14 (0.02)	10.28 (0.04)	8.42 (0.03)	2.3	1.3	2.5	0.1

[a] ± standard deviation in brackets.

[b] Day of fecal immobilization, t=0 of fermentation stabilization period.

[c]75% of Macfarlane media carbohydrate removed.

[d]Original cranberry extract produced using microwave extraction and ultrafiltration.

[e]Extract produced from enzymatic hydrolysis of original cranberry extract produced using microwave extraction and ultrafiltration.

[f]Cranberry pomace washed with 70% ethanol to remove small molecular weight carbohydrates, dried and ground.

The results (Table 3.5) show that the cranberry pomace and its corresponding polysaccharide/polyphenol-enriched extract resulted in an increase in the total bacterial flora and caused a significant increase in the *Clostridium* cluster XIVa (*Blautia* spp., *Roseburia* spp., *Eubacterium rectale)*, members of the *Firmicutes* family. The use of the enzymatically-hydrolyzed extract also affected positively the growth of this *Clostridium* luster, but with lower extent as compared to the pomace and the extract. Indeed, *Clostridium* cluster XIVa encompasses bacteria that inhabit the mucosal layer of the gut that are largely responsible for the production of mucin and butyrate, which inhibit pathogen colonization (Eren et al., 2015). Patients with irritable bowel syndrome (IBS) often show decreased mucus production, implying that prebiotics that stimulate the growth of these bacteria could have positive health implications for IBS patients (Lopetuso, Scaldaferri, Petito, & Gasbarrini, 2013; Van den Abbeele et al., 2013). A study investigating the effects of a low fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) diet demonstrated a seven-fold reduction in the *Clostridia* XIVa cluster, demonstrating the importance of fermentable substrates for this group of bacteria (Halmos et al., 2015). *Roseburia intestinalis and E. rectale* are important bacteria in the *Clostridium* XIVa cluster that produces significant amounts of butyrate. Mucosal bacteria are also located more closely to epithelial cells, providing greater benefit to the host immune system (McDowell, 2010). In addition, mucosal bacteria can move into the lumen due to shedding of the mucosa and migration of motile bacteria such as *Roseburia intestinalis and E. rectale*. Mucin producing bacteria are beneficial due to the fact that butyrate-producing members of the *Firmicutes* family can utilise mucin as a substrate (Lopetuso et al., 2013; Van den Abbeele et al., 2013). The growth of this cluster using cranberry extract, enzymatically-hydrolyzed cranberry extract or cranberry pomace as a substrate is very promising, as the growth of these bacteria would increase mucus production and encourage the growth of butyrate producers of the *Firmicutes* phyla. Inulin and large chain arabinoxylans have been shown to encourage the growth of this phyla and lead to the production of mucin and increased butyrate; humanized rats fed with inulin demonstrated an increase in *Clostridium* cluster XIVa from 52.37% to 64.15% of the total bacterial community (van den Abbeele et al., 2011). Contrarily, in the present study, oligofructose did not demonstrate an increase in bacteria belonging to the *Clostridium* XIVa cluster. However, the results show that the fermentation of the cranberry pomace and oligofructose led to a significant increase in the population of the *Clostridia* cluster IV, namely *Clostridium leptum*. The growth of butyrogenic

bacteria in the *Clostridia* cluster IV increases the butyrate production that is primarily used as energy by colonocytes, but also has implications on colonic health such as influencing gene expression, anti-inflammatory properties, and prevention of ulcerative colitis and colorectal cancer (Lopetuso et al., 2013; Velázquez, Lederer, & Rombeau, 1997). Van den Abbeele *et al.* (2011) reported that arabinoxylans and inulin enhanced the growth of butyrate producers in *Clostridia* cluster IV. Contrary to the cranberry extract and its hydrolysate, cranberry pomace was most likely well fermented by the *Clostridia* cluster IV due to the presence of arabinoxylans in the cell wall, which is associated with hemicellulose and pectin

Significant changes in *Bifidobacterium* spp. growth was observed for both the enzymatically-hydrolyzed cranberry extract and oligofructose, with an increase equal to 0.6 log and 0.5 log, respectively. *Bifidobacterium* population did not change significantly for the cranberry extract (-0.1 log) nor for the cranberry pomace (0.2 log). Rossi *et al.* (2005) demonstrated that *Bifidobacterium* spp. were able to ferment fructooligosaccharides, but failed to grow on inulin. They suggested that the growth of *Bifidobacterium* spp. is highly dependent on the metabolic activities and carbohydrate degradation by other species in the colonic microflora, suggesting that hydrolysis of larger molecular weight compounds by other bacteria is necessary to provide smaller molecular weight carbohydrates that are fermentable by *Bifidobacterium* spp. The carbohydrates in the cranberry extract and the cranberry pomace may have been too large or not hydrolyzed sufficiently for use as an energy source by *Bifidobacterium* spp. Some studies demonstrate the ability of *Bifidobacterium* spp. to ferment galactooligosaccharides, rhamnogalacturonan I, and arabinogalactans, ultimately producing butyrate and lactate as a fermentation product (Daguet et al., 2016; Flint, Scott, Duncan, Louis, & Forano, 2012; Khodaei, Fernandez, et al., 2016; Pokusaeva, Fitzgerald, & van Sinderen, 2011).

Enterobacteria are symbiotic inhabitants of the gut, but also include pathogens. Both the cranberry extract and the enzymatically-hydrolyzed cranberry extract did not enhance the growth of the *Enterobacteriaceae* family. Cranberry pomace and oligofructose significantly increased the growth of the *Enterobacteriaceae* family. The insignificant change in the growth of *Enterobacteria* during fermentation of cranberry extract and enzymatically hydrolyzed cranberry extract may be attributed to the fact that the isolated carbohydrate may have not been readily fermentable by *Enterobacteria*, and/or may have been consumed competitively by other bacteria. The presence of

polyphenolic compounds in the extract may have contributed to this result. This reveals that the extracts from cranberry would not encourage the growth of pathogens from the *Enterobacteriaceae* family in the gut. Piglets fed sugar beet pomace and wheat bran as part of their diet showed the lowest counts of *Enterobacteria* compared to a control diet (Molist et al., 2009). Soybean fiber rich in galactose has been reported to enhance the growth of E. *coli* in pigs (H. Chen et al., 2014), and rhamnogalacturonan I from potato has also stimulated the growth of the *Enterobacteriaceae* family in the human fecal inoculum (Khodaei, Fernandez, et al., 2016).

The *Bacteroides/Prevotella* group did not undergo any significant changes during fermentation of all substrates, nor did *Enterococcus* spp. Tea extracts rich in phenolic compounds were shown to prevent the growth of *Bacteroides* spp. (Lee, Jenner, Low, & Lee, 2006), suggesting that the phenolic compounds present in the cranberry extract and enzyme hydrolyzed extract may have had an inhibitory effect on the growth of *Bacteroides* spp. *Bacteroides* is generally associated with hosts following a western diet, high in protein and fats, whereas *Prevotella* is found in hosts that follow more of a plant based diet. However, both are considered pro-inflammatory (Ley, 2016). Since the growth of these bacteria was not significant during fermentation of cranberry extract and enzyme hydrolyzed cranberry extract, we can infer further that the cranberry extracts may help increase the *Firmicutes: Bacteroides* ratio.

3.4.3.2 Short Chain Fatty Acid Production

Short chain fatty acids (SCFA) are metabolic end-products, produced from the bacterial fermentation of digestion-resistant substrates, such as plant cell wall material, that serve to protect the colonic environment from pathogen colonization and act as a source of energy for epithelial cells in the colon (Tabernero & Gómez de Cedrón, 2017; Velázquez et al., 1997). Table 3.6 shows the SCFA profile produced at the end of each fermentation. Total SCFA concentrations were in the common range of 50-200 mM (Louis & Flint, 2017).

The main predominant SCFAs produced during fermentation of each substrate were acetic acid, propionic acid, and butyric acid. This is concurrent with several studies on the production of SCFAs by multiple species of bacteria in the gut (Daguet et al., 2016; Duncan et al., 2004; Gómez et al., 2016; Ríos-Covián et al., 2016; Yang, Martínez, Walter, Keshavarzian, & Rose, 2013). The results also show that the concentrations of acetate produced upon the fermentation of the

cranberry polysaccharide/polyphenol enriched-extract and the enzymatically hydrolyzed extract (76.05 mM and 75.60 mM, respectively) were higher as compared to those generated upon the fermentation of the cranberry pomace and oligofructose (69.76 mM and 68.98 mM, respectively). Similarly, it has been reported that pectins from various sources and arabinogalactan tend to produce greater amounts of acetic acid compared to fructooligosaccharides and inulin (Cantu-Jungles, Cipriani, et al., 2017; Daguet et al., 2016; Gullon et al., 2015; Khodaei, Fernandez, et al., 2016; Min et al., 2015). Indeed, acetate is important for the growth of many bacterial species in the colon and can be converted into butyrate by *Roseburia* spp. (Duncan et al., 2004). Furthermore, the increase in the population of bacteria in the *Clostridia* XIVa cluster after fermentation of cranberry extracts (Table 3.6; Δlog bacteria of 1.3-2.5) may have favoured the conversion of acetate to butyrate, resulting in similar butyrate concentration than that generated upon oligofructose fermentation. The higher concentration of *Bifidobacteria* spp. during oligofructose fermentation (7.05 Δlog cells/ml; Table 3.5) explains the higher concentration of butyrate with this substrate. Higher concentrations of butyrate were produced using cranberry extract as carbon source as compared to apple bagasse flour (2.64 mM); however, the fermentation of rhamnogalacturonan I isolated from potato (31.3 mmol/L) induced more production of butyrate than the cranberry extract (Gullon et al., 2015; Khodaei, Fernandez, et al., 2016).

Similar to previous studies, the fermentation of oligofructose produced inferior amounts of butyrate (23.89 mmol/L) compared to acetate (68.98 mmol/L) (Cantu-Jungles, Cipriani, et al., 2017; Daguet et al., 2016; Yang et al., 2013). The fermentation of inulin produced 26.07 µmol butyrate/ 100 mg carbohydrate compared to 183.75 µmol acetate/ 100 mg in a study performed using fecal bacteria (Yang et al., 2013). A similar trend was reported by another study involving the fermentation of inulin, where 14 µmol butyrate/ 50 mg carbohydrate versus 42.9 µmol acetate/ 100 mg was produced (Cantu-Jungles, Cipriani, et al., 2017). Daguet *et al.* (2016) demonstrated that while proximate colon concentrations of buyrate and acetate were similar for inulin (approximately 45 mmol/L), but in the distal colon acetate concentration was higher than butyrate (approximately 55 mmol/L compared to 45 mmol/L, respectively). Gomez *et al*. (2016) showed that fructooligosaccharides produced the highest amount of butyrate compared to pectic oligosaccharides isolated from sugar beet and lemon peel. The same trend was observed for inulin versus arabinoxylans, guar gum, resistant starch, pectin and β – glucan (Yang et al., 2013).

Table 3.6. Short chain fatty acid production during fermentation of various carbohydrate sources.

	Concentration[a]				
	25% CHO[c]	**Cranberry Extract[d]**	**Enzyme Hydrolysis Extract[e]**	**Cranberry Pomace[f]**	**Oligofructose**
Acetic Acid	65.36 (0.86)	76.05 (1.73)	75.6 (4.46)	69.76 (2.05)	68.98 (0.95)
Butyric Acid	24.54 (1.6)	23.09 (1.73)	19.96 (0.3)	19.36 (1.19)	23.89 (1.64)
Iso-Butyric Acid	5.9 (0.23)	5.52 (0.13)	5.5 (0.16)	5.72 (0.47)	5.21 (0.24)
Iso-Valeric Acid	10.43 (0.24)	9.67 (0.16)	9.68 (0.04)	9.77 (0.24)	8.42 (0.52)
Propionic Acid	30.97 (0.54)	41.05 (0.22)	39.83 (0.57)	32.11 (0.44)	51.06 (1.01)
Valeric Acid	5.86 (1.14)	3.22 (0.52)	9.1 (0.2)	8.77 (0.26)	4.2 (0.18)
Total SCFA	143.05	158.6	159.67	145.49	161.75

[a]Concentration expressed as mmol/L of fermentation media.

[b]Day 16 of stabilization period.

[c]MacFarlane media containing 25% of total carbohydrate.

[d]Original cranberry extract produced using microwave extraction and ultrafiltration.

[e]Extract produced from enzymatic hydrolysis of original cranberry extract produced using microwave extraction and ultrafiltration.

[f]Cranberry pomace washed with 70% ethanol to remove small molecular weight carbohydrates, dried and ground.

[g]± standard deviation in brackets.

This is in agreement with the results determined in the present study, where oligofructose produced the greatest amount of butyrate compared to cranberry extract, enzymatically hydrolyzed cranberry extract, and cranberry pomace. The results (Table 3.6) also indicate that the highest propionic acid concentrations were generated upon fermentation of oligofructose, followed by that of the polysaccharide/polyphenol enriched cranberry extract. It is known that the populations of the *Bacteroidetes* family are capable of producing propionate (Louis & Flint, 2017; Ríos-Covián et al., 2016). However, the relatively stable populations of *Bacteroidetes* during the investigated fermentations do not seem to be responsible for the changes in propionate concentrations. The increase in propionate production during the fermentation of the cranberry extract and enzymatically hydrolyzed cranberry extract may be due to the increase in *Clostridia* cluster XIVa. *Blautia* and *Roseburia* (*Clostridia* cluster XIVa members) species that are capable of utilizing rhamnose and fructose to produce propionate (Louis & Flint, 2017). The cranberry extract and enzymatically hydrolyzed cranberry extract could have provided easier access to fermentable regions of rhamnogalacturonan I as opposed to cranberry pomace, since the extracts were generated/extracted from the cell wall. The rhamnogalacturonan I in cranberry pomace is trapped within the cell wall and intertwined with cellulose and hemicellulose, making it more difficult to access (Helene, Canteri, & Nogueira, 2012; A. W. Zykwinska, Ralet, Garnier, & Thibault, 2005). Propionic acid may have also been generated from the fermentation of arabinogalactans, a polysaccharide found in hemicellulose (Macfarlane & Macfarlane, 2003). The presence of hemicellulosic polysaccharides in the cranberry extract (Table 2.8) and the enzymatically hydrolyzed cranberry extract may have also contributed to the formation of propionate.

The production of valeric acid may have been resulted from the fermentation of phenolic compounds, as flavan-3-ols may be catabolized to valeric acid by some bacteria (Jandhyala et al., 2015; Tabernero & Gómez de Cedrón, 2017). The enzymatically hydrolyzed cranberry extract led to the generation of the highest concentration of valeric acid (9.1 mM) compared to the other products. This reveals that the enzymatic hydrolysis may have improved the availability of phenolic compounds as a substrate for fermentation. Branched-chain fatty acids (BCFA), shown in Table 3.6 as iso-butyric and iso-valeric acid, are often products of increased protein fermentation and are regarded as negative indicators of colon health. They are frequently observed in higher concentration as a result of diets with low indigestible carbohydrates (Halmos et al., 2015).

Fermentation of all substrates in the present study did not increase production of BCFA, further confirming the potentially positive implications of the two cranberry materials on colon health.

3.5 Conclusions

Cranberry extracts generated using microwave assisted extraction and enzymatic hydrolysis using Viscozyme®L as well as cranberry pomace and oligofructose were subjected to TIM-1 digestion and colonic fermentation. Simulated digestion of the cranberry extract revealed that polysaccharides recovered in the chymes (1.27% of original meal) and effluents (1.22% of original meal) originated from the homogalacturonan and rhamnogalacturonan I region of pectic polysaccharides. Rhamnose, arabinose, galactose were detected in significant proportions in the effluents (0.46%, 0.95%, and 4.13% of the total digestas, respectively) and in the chymes (0.1%, 0.31%, and 3.19% of the total digestas, respectively). Rhamnose, in particular, was not detected in the dialysates. The uronic acid content of the effluents and chymes was also in higher proportion compared to the dialysates, suggesting the resistance to digestion of the pectic polysaccharides in the cranberry extract. Large proportions of glucose (29.07%) were obtained in the dialysates after digestion, implying that glucose containing poly- and oligosaccharides are affected by gastric digestion. The hydrolysis of cell wall oligo- and polysaccharides ultimately resulted in the release of esterified polyphenolic compounds. The polyphenolic content was increased 2.3 fold after gastric digestion, which may also be attributed to the hydrolysis of larger polyphenolic compounds into monomeric polyphenols. These findings demonstrate that digestion increases the bioavailability of phenolic compounds and implies that they can reach the colon as a fermentation substrate for bacteria. The colonic fermentation of the cranberry extracts as well as cranberry pomace and oligofructose was performed using bacteria obtained from human fecal matter. *Clostridium* cluster XIVa, which encompasses important butyrate and mucin producing bacteria, increased significantly for cranberry extract, enzyme hydrolyzed cranberry extract, and cranberry pomace ($\Delta\log = 2.3$, 1.3, and 2.5, respectively). Overall, the growth of this bacterial cluster will lead to an increase in the *Firmicutes:Bacteroidetes* ratio. The enzymatically hydrolyzed cranberry extract and oligofructose increased the population of *Bifidobacterium* ($\Delta\log = 0.6$ and 0.5, respectively). We attribute the smaller molecular weight of these polysaccharides for encouraging the growth of *Bifidobacterium*. *Enterobacteriaceae* nor *Bacteroides/Prevotella* did not increase upon fermentation of cranberry extract and enzyme hydrolyzed cranberry extract. The short chain

fatty acids produced at the end of each fermentation period showed that cranberry polysaccharide/polyphenol enriched extract and the enzymatically hydrolyzed extract generated the highest amounts of acetate (76.05 mM and 75.60 mM, respectively). Acetate is an important growth substrate for many bacteria in the colon and can be converted to butyrate by *Roseburia* spp., demonstrating the importance of high production of acetate. Highest amounts of butyrate were produced during oligofructose fermentation (23.89 mM), which is attributed to the significant growth of *Bifidobacteria* spp. during fermentation of oligofructose. The increase in propionic acid during cranberry extract and enzyme-hydrolyzed cranberry extract (41.05 mM and 39.83 mM, respectively) is possibly due to the growth of *Clostridia* cluster XIVa cluster. Valeric acid was produced in greatest quantity by the enzyme hydrolyzed cranberry extract, which may be a result of more bioavailable phenolic compounds produced during enzymatic hydrolysis. This initial study provided insight to the influence of gastric digestion on cranberry polysaccharide/polyphenol enriched extracts and their potential to increase a healthy colonic microflora. Further studies in an in-vivo environment have the potential to further understand they implications of these extracts on the human colonic environment.

CHAPTER IV

GENERAL SUMMARY AND CONCLUSIONS

The ability to generate an extract rich in plant cell wall polysaccharides and polyphenolic compounds from cranberry pomace was investigated. This was achieved through the use of microwave-assisted extractions under acidic and alkaline conditions at two microwave powers of 36 and 72 W/g of cell wall material. A sequential acidic/alkaline extraction method was also investigated, where cranberry pomace was first extracted under acidic conditions, and then re-extracted using alkaline conditions. All extraction methods generated different profiles regarding the total phenolic compounds and the carbohydrate recovery, the yield, the monosaccharide profile, and the molecular weight distribution. Acidic microwave extraction produced yields of 3.37-4.96%, total carbohydrate recovery of 2.35-2.65%, and total phenolic compound recovery of 4.06-13.21%. Alkaline microwave extraction resulted in yields of 10.36-12.65%, total carbohydrate recovery of 4.73-7.84%, and total phenolic compound recovery of 51.76-106.7%. Sequential acidic/alkaline microwave extraction resulted in yields of 7.72-8.0%, total carbohydrate recovery of 4.20-5.27%, and total phenolic compound recovery of 51.06-52.22%. The use of the sequential acidic/alkaline method at 72 W/g generated the highest proportion of uronic acid (64.02%), while at 72 W/g acidic extraction generated the highest proportion of rhamnose. The recovery of arabinose and galactose-rich oligo/polysaccharides was achieved with the alkaline extraction at 72 W/g (20.81% and 14.75%, respectively). Molecular weight varied significantly between extraction methods. Acidic extraction at 72 W/g generated the highest proportion of polysaccharides in the 200-700 kDa range (66.5%). Acidic extraction at 36 W/g generated the highest amounts of oligosaccharides in the 1-10 kDa range (28.9%). As a result of these findings, the sequential acidic/alkaline microwave extraction method was chosen for further optimization due to the more exhaustive approach on the material.

The optimization of the sequential microwave-assisted extraction method was executed using response surface methodology. The experimental variables included sodium hydroxide concentration (0-2 M), microwave power (35-80 W/g cell wall material), pomace concentration (3.33-50.0 mg/ml), and time (1-6 min). The optimal conditions were determined as 1.51 M NaOH, 65 W/g, 16.33 mg/ml, and 4.73 min to generate responses of 28.65% extract yield, 10.56% recovery of carbohydrates, and 57% recovery of total phenolic compounds. The carbohydrate portion of the extract was composed of 38.10% uronic acids and 61.90% neutral monosaccharides.

The molecular weight distribution showed that 5.03, 12.05, 23.58 and 45.64% of polysaccharides were in the range of 2.5 kDa or less, 400-900 kDa, 5-15 kDa and 50-150 kDa, respectively.

The optimized extraction conditions were applied to produce a cranberry extract rich in carbohydrates and polyphenolic compounds. An enzymatically hydrolyzed extract was also produced using Viscozyme L®. The original extract contained 24% of 605.7 kDa, 49% of 122 kDa, and 25% of 9.4 kDa polysaccharides with 2% of 0.75 kDa oligosaccharides. The enzymatically hydrolyzed extract consisted of 60% of 3.5 kDa, 10% of 1.4 kDa and 10% 0.7 kDa oligosaccharides and 20% disaccharides. TIM-1 gastric digestion of the original cranberry extract demonstrated that phenolic compounds were released during digestion (2.3-fold increase) and that polysaccharides were hydrolyzed to oligosaccharides and oligomers. Recovered in the effluent and chymes was 22% of the original weight of the meal, while the remained was absorbed via the jejunum and ileum dialysates. The recovery of a large proportion of uronic acid in the chyme and effluent (50.75% and 61.21%, respectively) as well as the recovery rhamnose (1.99% and 0.81%, respectively) suggested that the pectic polysaccharides in the cranberry extract resisted gastric digestion. Significant proportions of glucose and galactose in the dialysates suggests that hemicellulosic polysaccharides and possibly arabinogalactans were susceptible to hydrolysis during digestion.

In-vitro, continuous fermentation of cranberry materials were evaluated using human fecal microorganisms. The cranberry polysaccharide/polyphenol enriched extract, the enzymatically hydrolyzed extract, and the cranberry pomace as well as oligofructose were assessed as fermentation substrates by human fecal bacteria. Results demonstrated that cranberry-based products influenced the growth of bacteria in the *Clostridia* cluster XIVa (Δlog bacteria = 2.3,1.3,2.5, respectively). Oligofructose and the enzymatically hydrolyzed extract increased the populations of *Bifidobacterium* significantly (Δlog = 0.5 and 0.6, respectively). *Enterobacteriaceae* underwent significant growth using cranberry pomace and oligofructose as fermentation substrates (Δlog = 1.5 and 0.5, respectively). The *Bacteroides/Prevotella* and *Bacteroides* groups did not increase, possibly as a result of the inhibitory effects of phenolic compounds in the extracts. Variable short-chain fatty acid profiles were generated by all substrates. Both the cranberry extract and the enzymatically hydrolyzed one produced comparable amounts of total short chain fatty acids (158.6 mmol/L and 159.67 mmol/L, respectively) compared to

oligofructose (161.75 mmol/L). Furthermore, oligofructose produced higher amounts of butyric acid (68.96 mM) and propionic acid (51.06 mM), while the cranberry extract and the enzymatically hydrolyzed cranberry extract produced higher amounts of acetic acid (76.05 and 75.60 mM, respectively). Higher production of acetate could imply increased production of butyrate, as acetate is converted to butyrate by some bacterial species in the colon.

The understanding of the polysaccharide profile and total phenolic content of cranberry pomace extracts generated using microwave-assisted extraction provide an insight to the cell wall structure of cranberry pomace and its use as a material for the production of value-added products. The effects on the modulation of the human fecal bacteria community will further help to valorize the use of food industry wastes rich in cell wall material as potential sources of prebiotic compounds.

Future research, involving cranberry pomace extracts generated using microwave-assisted extraction, should aim to investigate the *in-vivo* functional properties to better understand the implications on human health. The types and structures of phenolic compounds isolated from cranberry pomace using microwave-assisted extraction remain to be identified and characterized Their specific implications on the modulation of the metabolism of gastro-intestinal microorganisms should be investigated to further understand their role in human gastro-intestinal health.

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