PRODUCTION AND CHARACTERIZATION OF OLIGO- AND POLYSACCHARIDE-DERIVED FUNCTIONAL INGREDIENTS FROM CRANBERRY (*VACCINIUM MACROCARPON*) CELL WALL MATERIALS

by

Eugenio Spadoni Andreani

Department of Food Science and Agricultural Chemistry

McGill University, Montreal

August 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Food Science and Agricultural Chemistry

SUGGESTED SHORT TITLE

OLIGO AND POLYSACCHARIDES FROM CRANBERRY CELL WALL AND THEIR FERULOYLATION

ABSTRACT

The extraction of oligosaccharides from cranberry pomace, the fibrous by-product of juice production, was explored. The effects of two power settings (84.9 and 242.4 W/g) and alkali concentrations (0.25 and 0.5 M potassium hydroxide) on microwave-assisted extraction were tested. Highest yield of oligosaccharides (average 21.5%) was obtained at higher alkalinity. The extracts were enriched in pectic oligosaccharides with degree of polymerization between 7 and 10. Enzymatic hydrolysis was tested with a commercial endo-galactanase and four multi-enzymatic catalysts with varied levels of polysaccharide-degrading activities. Depol™ 740L and Pectinex® Ultra SPL had the highest yields (average 39.8%). All multi-enzymatic biocatalysts led to yields equal or higher than the microwave-assisted approach, while the yield obtained by endo-galactanase was lower. All extracts were enriched in glucose and with less galactose than the ones obtained by microwave approach. Most of the oligosaccharides generated by multi-enzymatic biocatalysts had a degree of polymerization between 2 and 5.

Cell wall polysaccharides from Stevens variety cranberries were extracted with a sequence of four aqueous solvents. Hot buffer extracted mainly pectic polysaccharides. Chelating agents extracted mainly homogalacturonan-rich polysaccharides and oligosaccharides. This extract had the highest yield (11% w/w of starting material). The main neutral structures in these two extracts were pectic arabinan and type II arabinogalactan. Pectic polysaccharides extracted with diluted alkali had the highest abundance of neutral branches and were associated with less hemicelluloses than the previous two. Their pectic neutral branches were characterized by the presence of more galactan. Concentrated alkali extract contained prevalently hemicellulose.

Pectic polysaccharide were isolated from chelating agents and diluted alkali extracts by anion exchange and gel filtration chromatography. A series of pectin-degrading enzymatic activities was used as a tool to obtain information on their structure. Different levels and distributions of methyl esterification in homogalacturonan were determined by comparing the galacturonic acid released by pectin lyase and endo-polygalacturonase. Debranching enzymes active on 1,5-linked arabinose and 1,4-linked galactose revealed important characteristic features, including the presence of pectic galactan and branched type I arabinogalactan, with a short backbone and abundant arabinan branches. The presence of galactoarabinan was also proposed.

The enzymatic esterification with ferulic acid of polysaccharides from chelating agents and diluted alkali extracts was investigated. *Humicola insolens* feruloyl esterase was immobilized on iminodiacetic acid-copper modified epoxy-polyacrylic supports. Specific esterification activity was assessed on raffinose in surfactantless microemulsions of water in n-hexane/butanone with different water contents. Immobilized enzyme was found to be most active in n-hexane:butanone:water mixture of 51:46:3 (v:v:v), while free enzyme showed lower specific activity with a maximum at 6% water. These conditions were used for the esterification of cranberry polysaccharides. Higher feruloylation (22.1 – 26.6% consumption of ferulic acid) of chelating agents-extracted polysaccharides was achieved with immobilized enzyme than with free enzyme, while immobilization didn't impact the esterification of diluted alkali-extracted polysaccharides.

The fermentability in anaerobic environment of the prebiotic polysaccharide inulin and of cranberry polysaccharides in native and feruloylated forms by two strains of the beneficial gut bacteria *Lactobacillus brevis* and *Bifidobacterium longum* was compared. All polysaccharides promoted the generation of abundant propionic acid, followed by acetic acid. Feruloylation negatively impacted bacterial growth, the effect being more marked on diluted alkali-based polysaccharides and for *L. brevis*.

RÉSUMÉ

L'extraction d'oligosaccharides à partir du marc de canneberge, un sous-produit de la production du jus de canneberge, a été explorée. Les effets de deux niveaux de puissance de micro-onde (84,9 et 242,4 W/g) et concentrations alcalines (hydroxyde de potassium 0,25 et 0,5 M) sur l'extraction assistée par micro-onde ont été testés. Le rendement en oligosaccharides le plus élevé (21,5% en moyenne) était obtenu avec l'alcalinité la plus élevée. Les extraits étaient enrichis d'oligosaccharides pectiques avec degré de polymérisation entre 7 et 10. L'hydrolyse enzymatique a été investiguée en utilisant endo-galactanase et quatre catalyseurs multienzymatiques avec des niveaux variés d'activités dégradant les polysaccharides. Depol™ 740L et Pectinex® Ultra SPL ont conduit aux rendements les plus élevés (39,8 % en moyenne). En comparaison avec l'extraction alcaline assistée par micro-onde, les biocatalyseurs multienzymatiques ont conduit à des rendements égaux ou supérieurs. Les extraits, enrichies en glucose, contenaient oligosaccharides avec degré de polymérisation entre 2 et 5.

Les polysaccharides de la paroi cellulaire des canneberges de la variété Stevens ont été extraits en utilisant une méthode séquentielle basée sur l'utilisation de quatre solvants aqueux. L'extrait obtenu en utilisant le tampon chaud contenait principalement des polysaccharides pectiques. Les agents chélateurs ont extrait des polysaccharides et des oligosaccharides riches en homogalacturonane. Cet extrait avait le plus haut rendement (11% p/p de matière de départ). Les principales structures neutres présents dans ces deux extraits étaient l'arabinane pectique et l'arabinogalactane de type II. La solution alcaline diluée a permis d'extraire des polysaccharides pectiques ayant la plus grande abondance en branches neutres, et associés à moins d'hémicelluloses que les deux extraits précédents. Les branches neutres pectiques étaient caractérisées par la présence de plus de galactane. L'extrait obtenu par la solution alcaline concentrée contenait principalement de l'hémicellulose.

Des polysaccharides pectiques ont été isolées des extraits, obtenus par un agent chélatant et par la solution alcaline diluée, par la chromatographie à échange d'anions suivie de celle à filtration sur gel. Les fractions récupérées ont été soumises à une série d'activités enzymatiques dégradant les polysaccharides, permettant d'obtenir des informations sur leur structure. Le niveau et la distribution du groupe d'ester méthylique d'homogalacturonane étaient déterminés en comparant l'acide galacturonique libérée par la pectine lyase et par l'endo-polygalacturonase.

Des enzymes agissant sur les branches, en particulier l'arabinose lié en 1,5 et le galactose lié en 1,4, révélaient la présence du galactane pectique et d'arabinogalactane de type I ayant une courte chaîne principale et des ramifications abondantes d'arabinane. La présence du galactoarabinane a également été proposée.

L'estérification enzymatique avec l'acide férulique des polysaccharides présents dans les extraits d'agents chélatants et d'alcali dilué, a été investiguée. La feruloyl estérase de *Humicola insolens* a été immobilisée sur des supports époxy-polyacryliques modifiés par acide iminodiacétique et cuivre. L'activité d'estérification spécifique a été évaluée dans des microémulsions composées de différentes teneurs d'eau en n-hexane/butanone. L'enzyme immobilisée a montré l'activité la plus élevée dans le mélange 51:46:3 (v:v:v) de n-hexane/butanone/eau. L'enzyme libre avait une activité spécifique plus faible, et l'activité maximale a été obtenue en présence de 6% d'eau. Ces conditions ont été utilisées pour l'estérification des polysaccharides. Une féruloylation plus élevée (22,1-26,6% de consommation d'acide férulique) des polysaccharides présents dans l'extrait d'agents chélateurs a été obtenue avec l'enzyme immobilisée, tandis que l'immobilisation n'a pas eu d'incidence sur l'estérification des polysaccharides présents dans l'extrait d'alcali dilué.

Les fermentabilités du prébiotique inuline et des polysaccharides de canneberge sous formes natives et feruloylées en milieu anaérobique par deux souches de bactéries intestinales bénéfiques *Lactobacillus brevis* et *Bifidobacterium longum* ont été comparées. Tous les polysaccharides ont favorisé la génération d'acide propionique abondant, suivi par l'acide acétique. La féruloylation a eu un impact négatif sur la croissance bactérienne, l'effet étant plus marqué sur les polysaccharides alcalins dilués et sur *L. brevis*.

STATEMENT FROM THE THESIS OFFICE

According to the regulation of the Faculty of Graduate Studies and Research of McGill University, Guidelines for Thesis Preparation include:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must conform to all other requirements of the "Guideline for Thesis Preparation" in addition to the manuscripts.

As manuscripts for publication are frequently very concise documents, where appropriate additional material must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reports in the thesis.

In general when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contribution of Authors" as a preface of the thesis.

When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

CONTRIBUTION OF AUTHORS

This thesis consists of the following chapters:

Chapter I provides a short introduction and describes the research rational as well as the main and specific objectives of the study.

Chapter II presents a literature review including the description of plant cell wall polysaccharides, their biological role and bioactive and technological properties. A description of selected oligosaccharides and their properties follows. Then, the procedures for the extraction and purification of polysaccharides, their conversion to oligosaccharides and the analytical techniques for their characterization are discussed. Finally, selected methods for the esterification of polysaccharides and oligosaccharides are briefly presented, with a focus on enzymatic approaches.

Chapter III to VI are presented in the form of manuscripts and have been or will be submitted for publication. The connecting statements provide the rationale linking the different parts of this study. Chapter III compares the extraction of oligosaccharides from cranberry pomace with microwave and multi-enzymatic approaches. Chapter IV reports the preparation of cranberry cell wall extracts by sequential application of aqueous solvents, and the characterization of the polysaccharides they contain in terms of monosaccharide composition and linkage. Chapter V is a focused analysis of selected fractions of cranberry pectic polysaccharides, purified from the extracts by anion exchange and gel filtration chromatography, through selective fragmentation by a sequence of single enzymatic activities. Chapter VI describes the enzymatic esterification of cranberry polysaccharide extracts with ferulic acid by feruloyl esterase, detailing the immobilization, determination of esterification activity in water-in-organic solvent microemulsion and the comparison of the fermentability of feruloylated and native polysaccharides by selected intestinal bacteria as compared to prebiotic inulin.

Chapter VII provides a general conclusion to the thesis with a summary of major findings.

Chapter VIII outlines the contribution of this study to the field and provides recommendations regarding future research on the characterization of structure and prebiotic activity of cranberry pectic polysaccharides.

Eugenio Spadoni Andreani, the author, was responsible for the experimental work and the preparation of the first draft of the manuscripts for publication and dissertation.

Dr. Salwa Karboune, the PhD student's supervisor, guided and supervised all research and critically revised the manuscripts prior to their submission.

Dr. Lan Liu, the second author of Chapter IV, contributed to the research and experimental work related to the glycosidic linkage characterization by mass spectrometry.

Mingqin Li, the second author of Chapter VI, contributed to the experimental work related to the chromatographic analysis of short chain fatty acids.

Dr. Jennifer Ronholm, the third author of Chapter VI, contributed to the microbiological tests by assisting with their design and allowing the use of the anaerobic chamber.

RESEARCH CONTRIBUTIONS

POSTERS

1. **Spadoni Andreani, E.**, Davis, E. & Karboune, S. Enzymatic and Microwave-Assisted Alkaline Extraction of Oligo/Polysaccharides from Cranberry Pomace Byproducts. Presented at IFT 2016.
2. **Spadoni Andreani, E.** & Karboune, S. Characterization of Cranberry Pomace Pectic Polysaccharides by Sequential Enzymatic Fragmentation -Extraction and Preliminary Composition Analysis-. Presented at Biotrans 2017.
3. **Spadoni Andreani, E.** & Karboune, S. Sequential Extraction and Enzymatic Fragmentation for the Determination of the Composition and Structure of Cranberry Pomace Pectic Polysaccharides. Presented at IFT 2018.
4. **Spadoni Andreani, E.** & Karboune, S. Enzymatic Approach to Characterization and Modification of Pectic Polysaccharides from Cranberry Pomace. Presented at Biotrans 2019.

PUBLICATIONS

1. **Spadoni Andreani, E**. & Karboune, S. (2020). Comparison of Enzymatic and Microwave-Assisted Alkaline Extraction Approaches for the Generation of Oligosaccharides from American Cranberry (*Vaccinium macrocarpon*) Pomace. Journal of Food Science 85, 2443-2451.
2. **Spadoni Andreani, E**., Liu, L. & Karboune, S. (2020). Extraction and Characterization of Cell Wall Polysaccharides from Cranberry (*Vaccinium macrocarpon* var. Stevens) Pomace. (Submitted).
3. **Spadoni Andreani, E**. & Karboune, S. (2020). Characterization of the Structural Features of Cell Wall Pectic Polysaccharides in Stevens Variety Cranberry Pomace by Sequential Extraction Approach and Using Highly Specific Pectin-Hydrolyzing Enzymes. (To be submitted).
4. **Spadoni Andreani, E**., Li, M., Ronholm, J. & Karboune, S. (2020). Biocatalyzed Generation of Feruloylated Polysaccharides from Cranberry and Characterization of their Prebiotic Properties. (Submitted).

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to everyone that offered precious contribution to this work and, in particular, many thanks go to my supervisor Dr. Salwa Karboune for her essential guidance.

I am grateful to my lab colleagues Dr. Sooyoun Seo, Dr. Nastaran Khodaei, Dr. Amanda Waglay, Dr. Andrea Hill, Dr. Juan Tamayo, Dr. Jin Li, Neeyal Appanah, Afshan Malick, Erin Davis, Lily Chen, Marina Nguyen, Amal Sahyoun, Mehdi Sirouspour, Elham Chidar and Pratibha Sharma for the support, help and encouragement they gave me. The contribution of the expertise of Dr. Lan Liu and dr. Tara Sprules to this work is acknowledged with deep thankfulness. A thank you to the interns Margot Naji and Ningyi Wang that assisted me in some experiments.

Big thanks go to my family, that supported from afar my endeavors in Canada.

Special thanks to my wife and colleague Mingqin Li with whom I had the pleasure to share this study experience and whose determination and kindness helped me through the difficulties. While the pandemic is keeping us apart physically, it cannot truly separate us! We shall see a brighter future, be reunited and safe.

TABLE OF CONTENTS

LIST OF TABLES

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

LIST OF FIGURES

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

NOMENCLATURE/ LIST OF ABBREVIATIONS

HG	Homogalacturonan
RGI	Type I rhamnogalacturonan
RGII	Type II rhamnogalacturonan
XGA	Xylogalacturonan
NMR	Nuclear magnetic resonance
HPLC	High performance liquid chromatography
AIS	Alcohol insoluble solids
OS	Oligosaccharide
XOS	Xylo-oligosaccharides
IMOS	Isomalto-oligosaccharides
MOS	Mannan-oligosaccharides
GOS	Galacto-oligosaccharides
FOS	Fructo-oligosaccharides
POS	Pectic oligosaccharides
Xyl	Xylose
Glc	Glucose
Fru	Fructose
Ara	Arabinose
Gal	Galactose
HPAEC	High performance anion exchange chromatography
PAD	Pulsed amperometric detector
RI	Refractive index
ESI	Electrospray ionization
MALDI	Matrix-assisted laser desorption/ionization
PMP	1-phenyl-3-methyl-5-pyrazolone
NOESY	Nuclear Overhauser Effect spectroscopy
COSY	Correlated spectroscopy
TOCSY	Total correlated spectroscopy
HSQC	Heteronuclear single-quantum correlation

HMBC	Heteronuclear multiple-bond correlation
SCFA	Short-chain fatty acids
KOH	Potassium hydroxide
HCl	Hydrochloric acid
rpm	Rotations per minute
NaOH	Sodium hydroxide
HPSEC	High performance size exclusion chromatography
NaCl	Sodium chloride
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerization
HB	Hot buffer extract
CH	Chelating agents extract
DA	Diluted alkali extract
CA	Concentrated alkali extract
GalA	Galacturonic acid
Rha	Rhamnose
Man	Mannose
PMAA	Partially methylated alditol acetates
DSS	Sodium 4,4-dimethyl-4-silapentan-sulfonate
DMSO	Dimethyl sulfoxide
GC-MS	Gas chromatography-mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
PPS	Pectic polysaccharides
EDTA	Ethylenediaminetetraacetic acid
MRS medium	de Man-Rogosa-Sharpe medium
IDA	Iminodiacetic acid
MOPS	3-morpholinopropane-1-sulfonic acid
PA	Prebiotic activity score
CFU	Colony-forming units
PTFE	Polytetrafluoroethylene

CHAPTER I. GENERAL INTRODUCTION

Cranberry (*Vaccinium macrocarpon*) is a perennial bog shrub native to North America that produces ovoidal berries about 2 cm long, with red or pink waxy cuticle, white pulp and a sour and bitter taste. Cranberries are cultivated mainly in eastern United States, eastern Canada and Chile. Due to their strong flavour, only about 5% of the fruits is used as fresh produce, while the rest is processed mostly into juice, but also dry raisin-like products, sauces and jams (Neto & Vinson, 2011). While the consumption of cranberry-based products holds cultural relevance in the United States and Canada, global interest and production of cranberry juice and extracts has greatly increased in the past decades because of the popularization of their health-promoting properties: cranberry has in fact been historically used for prevention and treatment of urinary tract infections by Native Americans (Locke, 2018), a use that has survived as home remedy in American society in the form of juice consumption and is now spreading worldwide. As cranberry juice production increases, the importance of finding applications for the solid by-product of this process, cranberry pomace, becomes more relevant.

Fruit pomaces are constituted of fibrous materials from fruit peduncle, seeds, exocarp, cuticle and parts of the pulp and are rich in cell wall macromolecules (cellulose, hemicelluloses and pectic polysaccharides, lignin and structural proteins), carotenoids and polyphenols. While lignin and cellulose are the two most abundant biological polymers and their extraction is easier from other biomasses such as wood pulp, fruit pomaces are cost-effective starting materials for the extraction of pectic polysaccharides (apple, orange), phenolic compounds (apple, berries) and carotenoids (tomato). Carotenoids and polyphenols are employed as natural colorants and food preservatives because of their strong antioxidant properties (Giusti & Wallace, 2009; Kumar & Sinha, 2004). Pectic polysaccharides and less commonly hemicelluloses are used as gelling agents and emulsion stabilizers in beverages (Abid et al., 2017; Dickinson, 2009), as well as to improve the texture and increase the fiber content of solid foods. Furthermore, pectic polysaccharides have been found to possess *in vitro* prebiotic activity (Tingirikari, 2019), and the oligosaccharides that can be obtained from hemicelluloses and pectic polysaccharides also possess health-promoting properties, such as anti-inflammatory (H. Tan et al., 2018), prebiotic (Moon et al., 2015; Rabelo et al., 2006; Vigsnæs et al., 2011), antimicrobial (P. Li et al., 2016) and anti-biofilm effects (Sun et al., 2015). Feruloylated oligosaccharides are water soluble esters of ferulic acid, an abundant cell wall phenolic acid.

These oligosaccharides, obtained from the hydrolysis of naturally occurring feruloylated polysaccharides such as cereals' arabinoxylan (Rudjito et al., 2019) and the pectin of beet and other Caryophyllales (Ralet et al., 1994; Sato et al., 2013), have shown anti-inflammatory effects (Fang et al., 2012) and improved antioxidant properties (Ohta et al., 1994). A different prebiotic activity compared to similar non-feruloylated compounds was found for wheat-derived feruloylated oligosaccharides (Gong et al., 2019), while the beet-derived ones appeared to have activity analogous to the non-feruloylated (Holck, Lorentzen, et al., 2011). The generation of feruloylated mono, di and oligosaccharides through enzymatic activity has been achieved by employing feruloyl esterase (EC 3.1.1.73) (Tamayo-Cabezas & Karboune, 2019), and this approach could be extended to polysaccharides, with the potential of modulating their functional properties.

While some cranberry pomace is currently commercialized by fruit processors and nutraceuticals companies as functional ingredient (source of fiber and antioxidants) for pet and human nutrition, most of it cannot be directly utilized because its strong color, taste and fibrous consistency make it difficult to integrate in food formulations, and its low pH and protein content make it unsuitable as livestock feed and detrimental to the soil quality if disposed in landfills (Vattem & Shetty, 2002). Therefore, cranberry pomace is instead utilized as a source of phenolic compounds to be used as antioxidants (Ablett, 2012), colorants (European Food Safety Authority number E163, permitted by Health Canada), and for their health-promoting properties (Clydesdale et al., 1979; Harrison et al., 2013; White et al., 2010a; Woo et al., 1980). Cranberries are in fact rich in type-A proanthocyanidins, that possess *in vitro* and *in vivo* anti-biofilm effect on pathogenic bacteria (Burger et al., 2002; Howell et al., 2005; Tufenkji et al., 2010).

While cranberry phenolic compounds have been extensively characterized, limited studies have been conducted on the cell wall polysaccharides, despite their abundance in pomace (Holmes & Rha, 1978; Park & Zhao, 2006), and their isolation remains scarcely investigated. Among the cell wall polysaccharides, pectic polysaccharides are the most structurally complex, as they feature both linear and branched "hairy" regions, with a variety of glycosidic residues and linkages (Bonnin et al., 2014). Linear regions are polymers of partly esterified galacturonic acid, either without decoration (homogalacturonan) or decorated with monomers of xylose (xylogalacturonan).

Hairy regions can be classified as either rhamnogalacturonan type I or II. The first is composed by a backbone of galacturonic acid and rhamnose residues, branched with diverse neutral sugar polymers including arabinose (arabinans), galactose (galactans) or mixed (arabinogalactans) (Khodaei & Karboune, 2013). The second, rarer, consists of a very conserved sequence of four short branches containing rhamnose, apiose, galactose, arabinose and other uncommon glycosyl residues, with a poly-galacturonic acid backbone (Bar-Peled et al., 2012). Different species and tissues contain pectic polysaccharides with different degrees of esterification, abundance and branching of hairy regions. A better understanding of the amount, extractability, structural properties and characteristics of cranberry cell wall polysaccharides would facilitate the design of processes for further valorizing cranberry pomace and reducing the waste associated with fruit processing by using it as source of functional polysaccharides and polysaccharide-derived ingredients.

The main objective of this research work was the investigation of selected approaches for the isolation of pectic polysaccharides and oligosaccharides from cranberry pomace, their structural characterization and the assessment of the possibility of modifying them by esterification with ferulic acid to modulate their properties as functional food ingredients. It is divided into the following specific objectives:

1) Characterization of cranberry pomace and evaluation of selected extraction techniques for the isolation of pectic polysaccharides and oligosaccharides from cranberry cell wall.
2) Quantification and characterization of the structural features of cell wall polysaccharides extracted from cranberry pomace by sequential extraction approach.
3) Analysis of glycosidic linkage in purified pectic polysaccharide fractions by enzymatic fragmentation.
4) Investigation of the applicability of feruloyl esterase, free or immobilized, for the esterification of polysaccharide extracts with ferulic acid
5) Evaluation of the fermentability of native and feruloylated cranberry polysaccharides by strains of beneficial gut bacteria.

CHAPTER II. LITERATURE REVIEW

2.1. Introduction

Plant cell wall is an abundant and complex material, exhibiting great variability depending on the source. Several compounds can be extracted from it, and the ability to convert it into value-added chemicals is key for reducing waste in several industrial processes, from paper production to fruit juice. This literature review will cover the general properties of the cell wall polysaccharides, with focus on the information available about the fruit cell wall in cranberry (*Vaccinium macrocarpon*) and other plants of its family. Methods for the extraction of polysaccharides and the synthesis of oligosaccharides will then be presented, with the main applications as functional food ingredients of these compounds and selected methods for their analysis. Modification of polysaccharides to modulate their properties will also be discussed.

2.2. Polysaccharides in the plant cell wall structures

Seed plants possess an extracellular matrix composed of three structures: middle lamella, primary cell wall, and secondary cell wall (Evert, 2006).

The middle lamella is the most peripheral element and is synthesized immediately upon mitosis. It consists of a single layer of adhesive pectic polysaccharides (linear and branched polymers of galacturonic acid) that gives continuity to the extracellular matrix by joining the primary cell walls of adjacent cells (Zamil & Geitmann, 2017).

The primary cell wall is composed of water, polymers (containing about 70% water and 30% of solids (Monro et al., 1976)) and minerals. The polymers are in turn composed of about 90% carbohydrates: cellulose microfibrils, hemicelluloses (also called cross-linking glycans) and pectic polysaccharides; the rest are structural glycoproteins. The dry weight content of structural glycoproteins and minerals in the primary cell wall was reported to be in the range of 2 to 10% and 1 to 5%, respectively (Rose, 2003).

Polymers are interconnected, forming a network: cellulose microfibrils form hydrogen bonds with hemicelluloses, pectic polysaccharides and glycoproteins; hemicelluloses and pectic polysaccharides are connected by covalent bonds to each other, and some glycoproteins are covalently bound to pectin (Fry, 2010; Zykwinska et al., 2005).

Enzymes with polysaccharide modifying activity, such as pectin methylesterase, are also present in the primary cell wall and play a role during plant tissue growth and differentiation (Buchanan et al., 2015). In most plants the primary wall consists of hemicelluloses -mainly composed of xyloglucans and glucomannans- and cellulose in similar amounts, as well as 20-35% of pectic polysaccharides. In cereals (Poaceae) the content of pectic polysaccharides is lower (<10%) and the preponderant hemicelluloses are arabinoxylans, while the xyloglucans are lesser and have a lower xylose content (Fry, 2010). Cereals also contain non-cellulose β-glucans in their primary wall (Burton & Fincher, 2009).

The secondary cell wall is developed by some cells of the vegetal organism upon reaching their final dimensions. Akin to the primary cell wall, this structure is mainly composed of cellulose microfibrils and hemicelluloses, most notably xylans. However, the content of pectic substances is very limited or null and there are no structural proteins or enzymes. Instead lignin, a complex phenolic alcohol polymer, is generally formed in the spaces between the cellulose fibers, making the secondary cell wall a rigid and almost impermeable structure (Meents et al., 2018).

2.3. Pectic polysaccharides

Pectic polysaccharides are macromolecules characterized by the presence of linear regions rich in galacturonic acid residues and 'hairy' regions with branches composed of neutral sugars (Coenen et al., 2007; Holck, Hjernø, et al., 2011). The various regions are covalently bound together (Bonnin et al., 2014) by glycosidic linkages. In seed plants, three main regions have been identified: homogalacturonan (HG) and two rhamnogalacturonans, type I (RGI) and type II (RGII). In certain plants, such as apples, soybean and potatoes, substituted HG has been described; the main example of this fourth type of structure is xylogalacturonan (XGA) (Schols et al., 1995; Joris Zandleven et al., 2007). Pectic polysaccharides are mostly composed by HG and RGI (both ~40% of pectic polysaccharides dry weight), followed by RGII (<15%) (Schindler, 1998).

Pectic polysaccharides can be distinguished according to solubility (Sucharipa, 1924): protopectin is tightly integrated in the network with the other polysaccharides, making it insoluble in water. During fruit ripening, enzymes hydrolyze the covalent bonds in the network, liberating pectin (Paniagua et al., 2014). Treatment with acids and heat also causes this separation.

Pectin is the portion of pectic polysaccharides that is not covalently bound to hemicelluloses and is therefore water soluble. Pectin displays colloidal properties when mixed to water, contributing to the cell wall viscosity. The carboxylic acid of galacturonic acid contained in pectin can be esterified with methyl groups, and higher degree of methyl esterification corresponds with a higher jelling power (Yen & Lin, 1998). Pectin with an extremely low degree of methyl esterification (<5%), sometimes referred to as pectic acid, is stabilized in the cell wall by multiple ionic bonds with polyvalent metal ions (Arzani et al., 2011; Mravec et al., 2017) and can be extracted with chelating agents. Pectic acid is water soluble as pectate polyanion, while not soluble in more acidic solutions (Khan et al., 2015). During fruit ripening, the endogenous pectin esterase enzyme catalyzes the removal of methyl esters from pectins, increasing the amount of pectic acid.

2.3.1. Homogalacturonan

HG is a linear polymer of α-(1,4)-D-galacturonic acid residues. A common modification of the residues is the formation of methylesters at the carboxylic group. Less frequent modifications are the formation of acetylesters at C2 and C3 (Grasdalen et al., 1988). Table 2.1 shows the degrees of methyl esterification and acetylation of pectic substances in fractions of commercial pectin sources and in berries. As it is apparent from sugar beet and apple-derived materials, even pectic polysaccharides from the same source can display extremely different values. This depends from the presence in different parts of the plant and growth stages of different levels of esterification, as well as from the experimental conditions and analytical method applied.

2.3.2. Xylogalacturonan

XGA consists of a backbone of α-(1,4)-D-galacturonic acid, branched at C3 and C2 with monomeric (Kikuchi et al., 1996) and dimeric (J. Zandleven et al., 2006) β-D-xylose. While generally considered as linked to HG by glycosidic bond between galacturonic acid residues (Bonnin et al., 2014), it has been shown to also occasionally occur as a branch of RGI (Oechslin et al., 2003; Schols et al., 1995).

Table 2.1 Degrees of esterification of different plant materials.

Source	Material formulation	Analytical method	DM (%)	DA (%)	References
Sugar beet	Dried flakes	NMR	29.1	18.0	Müller-Maatsch et al. (2014)
Sugar beet	Fresh pulp	HPLC	52.0	34.0	Renard and Thibault (1993)
Apple	Press cake	NMR	40.0	5.6	Müller-Maatsch et al. (2014)
Apple	Fresh pulp	HPLC	63.0	14.0	Renard and Thibault (1993)
Orange	Commercial pectin extract	HPLC	72.0	<1.0	Axelos et al. (1989)
Grape	Pomace	NMR	5.0	1.0	Müller-Maatsch et al. (2014)
Black currant	Fresh pulp	HPLC	91.0	8.0	Hilz et al. (2005)
Bilberry	Fresh pulp	HPLC	87.0	2.0	Hilz et al. (2005)

DM: degree of methyl esterification; DA: degree of acetylation; NMR: Nuclear magnetic resonance; HPLC-RI: High performance liquid chromatography-refractive index detector.

2.3.3. Rhamnogalacturonan type I

RGI is a family of pectic polysaccharides that contain a backbone of the repeating disaccharide [α-L-rhamnose-(1,4)-α-D-galacturonic acid-(1,2)] (Bonnin et al., 2014). The galacturonic acid residues display the same acetylations found in HG (Figure 2.1 A). The backbone is branched at the C4 of rhamnose residues, with monomers and polymeric side chains of L-arabinose and D-galactose (Prade et al., 1999). Several types of side chains have been identified, the most common being galactan, arabinan, and two types of arabinogalactan. Galactan is a relatively linear polymer of β-(1,4)-D-galactose (Figure 2.1 B). Arabinan has a backbone of α-(1,5)-L-arabinose, branched at C2 or C3 with α-L-arabinose monomers and dimers (Figure 2.1 C). Arabinogalactan type I has a backbone of β-(1,4)-D-galactose, branched at C3 with α-L-arabinose or arabinan (Figure 2.1 D), while arabinogalactan type II has a backbone of β-(1,3)-D-galactose, branched at C6 with polymers of β-(1,6)-D-galactose, in turn branched at C3 by L-arabinose (Figure 2.1 E). Some cell wall glycoproteins possess arabinogalactan type II as their carbohydrate moiety, and are linked to the pectic polysaccharides by the glycosidic bond between this side chain and RGI (L. Tan et al., 2013).

Several other structures, such as XGA (Schols et al., 1995) and arabinan with galactose-containing branches (Yapo, 2011; Yu et al., 2015) have been described as possible side chains in some plant materials, giving great complexity and variability to these polysaccharides.

The number, the length and the branching of these side chains greatly vary among the different cell types and during the plant development (Mohnen, 2008; Peña & Carpita, 2004). Experiments on the development of plants genetically modified to express RGI degrading enzymes showed that altering backbone and arabinans causes severe morphological changes (Oomen et al., 2003), but the exact biological roles of RGI remain unclear.

The amount of galactan seems to be positively correlated to tissue firmness, suggesting a possible effect on cell wall porosity, as suggested by a study comparing the mobility of RGI galactan and arabinan branches in two apple varieties with different firmness (Ng et al., 2014). Galactan branches were more abundant and/or possessing greater structural mobility in the fruits from the firmer variety. In both varieties, arabinan side chains had higher mobility than galactan. These findings show that the effect of RGI structure on cell wall firmness is not simply due to its physical properties, but more likely involves its integration with the rest of the cell wall matrix.

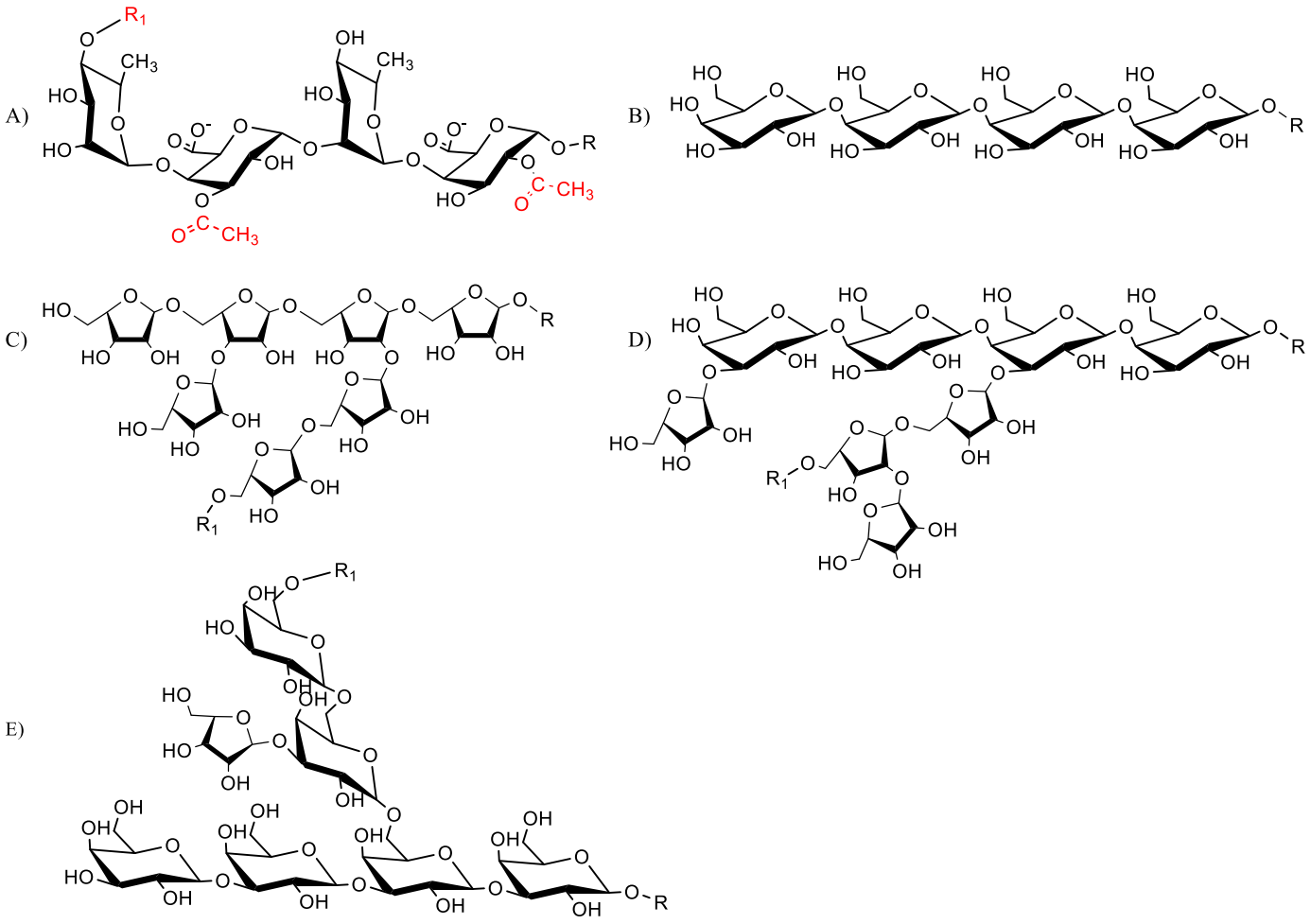

Figure 2.1 Common rhamnogalacturonan type I structures. A) Backbone of RGI; B) Galactan sidechain; C) Arabinan sidechain; D) Arabinogalactan type I sidechain; E) Arabinogalactan type II sidechain.

2.3.4. Rhamnogalacturonan type II

RGII consists of backbones of 8-10 α-(1,4)-D-GalA residues, branched with four side chains whose structures are strongly conserved across the plant kingdom (Ridley et al., 2001). The side chains include 12 different monosaccharide residues with 20 types of linkage. Six of these sugars are rarely found anywhere else in the cell wall. Like RGI, RGII is covalently bound to the other regions of the pectic polysaccharides, from which it can be enzymatically released as a free water-soluble form. It has been demonstrated that, in the cell wall, RGII exists as a dimeric ester, formed by the reaction of two apiose residues with a boron atom. Some evidence indicates that these structures may play a role in the structural stability of the cell wall (Funakawa & Miwa, 2015; Kobayashi et al., 1996).

2.4. Cellulose

Cellulose is a polysaccharide consisting of a linear chain of 100 to more than 15,000 β-(1,4)-D-glucose units (Zhang & Lynd, 2004). Because of their structure, cellulose molecules can form several intermolecular hydrogen bonds with the adjacent polysaccharides, including the RGI branches of pectic polysaccharides (Zykwinska et al., 2005), hemicelluloses and other cellulose molecules. Therefore, biosynthesized cellulose self-assembles into microfibrils (Heredia et al., 1995), which are crucial for the cell wall rigidity and the integrity of the plant cell. Plants in the family Poaceae (cereals) contain, along with cellulose, other linear and unsubstituted polymers of D-glucose, which contain not only the β-(1,4), but also the β-(1,3) linkage. The irregular distribution of the two linkage types confers them an amorphous structure, which is water soluble and gel-like (Burton & Fincher, 2009).

2.5. Hemicelluloses

Hemicelluloses are a diverse group of polysaccharides characterized by β-(1,4) linked backbones, but shorter and without the crystalline characteristics of cellulose. In the cell wall, these polymers are associated with cellulose and are at least in part covalently bound to RGI (Brett et al., 2005; Popper & Fry, 2008). The depolymerization and the solubilization of hemicelluloses are connected to the fruit ripening process (Cutillas-Iturralde et al., 1994; Lahaye et al., 2012).

The most abundant hemicelluloses in dicotyledons are the xyloglucans (Figure 2.2 A), characterized by a backbone of D-glucose residues and branches of α-(1,6)-D-xylose (Levy & Staehelin, 1992), followed by the heteromannans and heteroxylans. Heteromannans include glucomannans, with a backbone of D-mannose and D-glucose and short branches (e.g. β-L-arabinose, α-D-galactose, β-D-galactose-(1,2)-α- D-galactose) linked to the C6 of the mannose residues (Schröder et al., 2001; Sims et al., 1997) (Figure 2.2 B) and galactomannans, backbones of D-mannose with branches of α-(1,6)-D-galactose (Figure 2.2 C). Heteroxylans have a backbone of D-xylose, D-glucuronic acid and other residues, branched with β-(1,2)-D-glucuronic acid and α-(1→3)-L-arabinose (Figure 2.2 F). The cell wall of cereals is characterized by the abundance of a different class of hemicelluloses, the xylans, among which are glucuronoxylans and arabinoxylans. The first possess a backbone of D-xylose with branches of β-(1→2)-D-glucuronic acid, which is partly methylated on C4 (Rosell & Svensson, 1975) (Figure 2.2 D). Arabinoxylans have the same xylose backbone, but present branches of α-(1→3)-L-arabinose (Figure 2.2 E).

2.6. Cell wall composition of berries of the family Ericaceae

Berries are fruits that are characterized by their entirely soft pulp. This is due to the fact that the few lignified cells that are present do not form a shell in the endocarp around the seeds, but are instead scattered (Blaker & Olmstead, 2014). In the ripe fruit, the vascular tissue contains very low amounts of lignin, while the remaining tissues are composed only of primary cell walls, without lignin. The single-layer epidermal tissue secretes a thin waxy membrane, the cuticle, on the surface of the berry (Rosenquist & Morrison, 1988).

The family Ericaceae comprises of many shrubs and small trees of economic relevance, such as the ornamental azalea and rhododendron as well as the edible berries of the genus *Vaccinium*: blueberry, cranberry, bilberry, lingonberry and huckleberry.

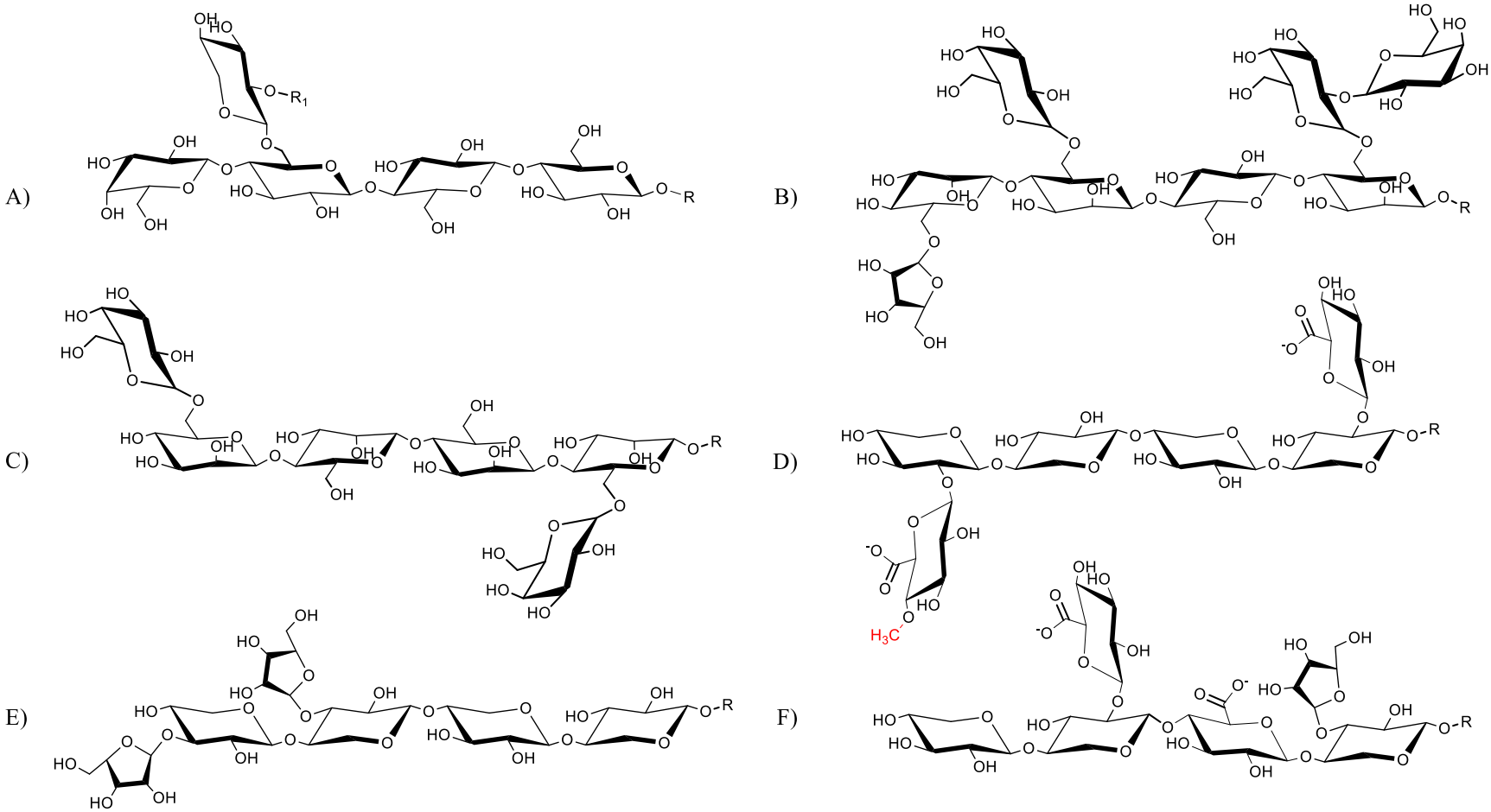

Figure 2.2 Hemicellulose structures. A) Xyloglucan; B) Glucomannan; C) Galactomannan; D) Glucuronoxylan; E) Arabinoxylan; F) Heteroxylan.

Most of the studies concerning cell wall composition of the fruits of Ericaceae were performed on blueberries, which are the most widespread cultivated species and on bilberries, which are rarely cultivated but are important fruits in Scandinavian cultures. Five blueberry cultivars from two species, *Vaccinium virgatum* (rabbiteye blueberry) and *V. corymbosum* (highbush blueberry), were analyzed to determine their cell wall composition (J. L. Silva et al., 2005); an average of 0.548 g of galacturonic acid, indicative of the presence of pectic polysaccharides, per 100 g of fresh fruits was reported. 65% of the pectic polysaccharides were alkali soluble polysaccharides (protopectin), 35% were oxalate soluble (pectic acids) and 4% were water-soluble (pectin). Although both species contained equal amount of total pectic polysaccharides, *V. virgatum* contained more protopectin and less pectin than *V. corymbosum*. Moreover, *V. virgatum* had a higher fiber content than *V. corymbosum* (17.94% versus 9.66% of dry weight, measured with neutral detergent technique), and its fibers had a 37:35:29 lignin: cellulose: hemicellulose ratio, while in the second species the ratio was 49:34:17.

A study on the relation between blueberry ripening and cell wall modifications showed that the main change is the solubilization of pectins from protopectin, which happened in the first stage of ripening (Vicente et al., 2007). Hemicelluloses were also gradually depolymerized. The fruit's cell wall was found to be rich in xylose and arabinose (50.5% and 30.75% of the non-glucose neutral sugars at ripeness), which leads the authors to hypothesize an unusually high presence of xylans.

The cell wall polysaccharides of *V. myrtillus* fruits (bilberries) have been characterized as well (Hilz et al., 2005). The dry weight of whole berries was 12.4%, and their alcohol insoluble solids (AIS) content was 3.8%. AIS, which contained the cell wall polymers, were sequentially extracted without breaking the seeds, and the sugar composition of the fractions was analyzed. Half of the total galacturonic acid was recovered in the first two fractions (extractions with hot buffer and chelating agents) together with arabinose, galactose and, to a lesser extent, glucose, glucuronic acid, xylose and rhamnose. In these fractions the molar ratio of arabinose to galactose was close to 1, and their ratio with galacturonic acid was about 0.12:1. The degree of acetylation for fraction 1 and 2 was 2 and 3%, respectively, whereas the degree of methyl esterification was 87% for fraction 1 and 77% for fraction 2.

Another 15% of galacturonic acid was extracted in fraction 3 (diluted alkali extraction) together with a relatively high concentration of the sugars associated to RGI. In this case, the molar ratio of arabinose and galactose to uronic residues was 0.29, and the two neutral sugars were equimolar. The last extracted fraction (concentrated alkali extraction) contained hemicellulose. In this fraction, the content of glucose was found to be much higher than that of xylose. In addition, this fraction contained lower amounts of all the characterized sugars. In a later study (Hilz et al., 2007), the structure of bilberry xyloglucans was established, identifying the presence of β-xylose branches. In the residual fraction, which contained the whole seeds, glucose, xylose and galacturonic acid were the major sugars. The majority of total glucose and xylose was found there, due to the poor extractability of hemicellulose from the lignified seeds. RGII was quantified in the pectic fractions of bilberry, and was estimated to represent 2% w/w of the total polysaccharides contained in AIS (Hilz et al., 2006).

2.7. Cell wall composition of cranberry (*Vaccinium macrocarpon*) fruit

A very limited number of studies on the cell wall composition of cranberry fruit is found in the literature. Total solids from pressed cranberry puree were characterized in an outdated study (Holmes & Rha, 1978). The authors found that upon the subjection of the puree to four centrifugations in water all the cell wall components, excluding cellulose, were partially removed from the solids with every wash. An unspecified majority of the undetermined total solids (26.2% w/w of total dry weight) was reportedly composed of hemicelluloses; the composition of the remaining 73.8% was 18.1% cellulose, 15.8% pectin, 2.17% crude proteins, 2.90% crude fat, 1.33% ash, 0.15% starch and 24.5% of neutral sugars, organic acids and anthocyanins.

The soluble and insoluble fractions of dietary fiber of canned cranberry sauce have also been analyzed (Marlett & Vollendorf, 1994). In the soluble fiber, uronic acids represented 71.4% of the dry weight, and the remaining 28.6% of neutral sugars was divided as follows: arabinose 30%, glucose 28%, galactose and rhamnose 21%, xylose 11% and mannose 10%. The dry weight percentage of insoluble fiber components was 60% neutral sugars, 5% uronic acids and 35% lignin. The major neutral sugar was glucose (64%), but the presence of small amounts of the other sugars was also detected.

More recently, cranberry pomace obtained from the processing of commercial cranberry juice was characterized by proximate analysis (Park & Zhao, 2006; White et al., 2010b). In the first study, dried pomace had a composition of 85.8% carbohydrates, 8.2% crude protein, 1.2% crude fat and 0.8% ash. In the second study, the composition of dried pomace was 83% carbohydrates (74.6% dietary fiber, 8.4% non-fiber), 2.3% crude protein, 12.6% crude fat and 1.2% ash. The differences in values between the two studies can be attributed to a great variability of the material. In fact, the composition of fruits from the Ericaceae family has been shown to be highly dependent on several factors, including cultivar and ripeness (Silva et al., 2005; Vicente et al., 2007), which are hard to assess for industrially produced pomace.

One work conducted by Stückrath et al., (1998) on three different cultivars of the species (Stevens, Ben Lear and Pilgrim) provided a more focused study of cranberry's pectic polysaccharides. 54-67% of the pectic polysaccharides were found to be in the protopectin form, while only 10-18% were in the high-methoxy pectin form. The degree of methyl esterification was found to be 45-67% for protopectin and 28-44% for pectin. The degree in total extracted pectin spanned between 47.7 and 57.5%, depending on the cultivar.

2.8. Polysaccharide-based functional ingredients

Several polysaccharides, often in the form of polysaccharide-rich extracts, are added to foods as functional ingredients, meaning that their inclusion provides physicochemical or biological properties to the product. Starches are mixtures of amylose and amylopectin; they are widely used in the food industry for their physical properties. Natural starches from different sources and modified starches (e.g. lipophilic starch, de-branched starch) have in fact different texture forming, gelling, water retaining and emulsifying qualities (Taggart, 2004). Dietary fiber refers to all the organic compounds found in food that are resistant to stomach acidity and digestive enzymes. Most of the dietary fiber functional ingredients are polysaccharides. Some are derived from the plant cell wall material like modified celluloses, which have several applications such as stabilizers for oil in water emulsions (Kolanowski et al., 2004), and arabinoxylans, which may be added to the dough to reduce bread staling (Gray & BeMiller, 2003). Others, such as inulin, exudate gums and resistant starch are not part of the cell wall. Fructans are lightly sweet but not completely digestible.

Therefore they are used as low-calorie sweeteners in certain products, such as low-calorie chocolates (Rapaille et al., 1995). Guar gum and inulin are used to partly replace fats in bakery, meat and cheese products (Brummel & Lee, 1990). The organoleptic features are not altered by these substitutions.

Fibers of other origins are also mainly composed of polysaccharides: chitin (from fungi and crustaceans), alginates (from algae) and xanthan (from *Xanthomonas* bacteria) (Imeson, 1992). Agar and carrageen extracted from algae are employed as jellifying ingredients in puddings, while xanthan is commonly used to stabilize the emulsion of industrially produced yoghurt and ice cream. Polydextrose is a synthetic polysaccharide functional ingredient, used to increase the viscosity of beverages (Auerbach et al., 2012).

2.8.1. Technological properties of pectic polysaccharides

Pectic polysaccharides extracts are widely used to increase the viscosity of beverages. Fruit-derived pectin (from apple and *Citrus* sp.) is commonly added as a stabilizer in jams (Grigelmo-Miguel & Martín-Belloso, 1999). Soy soluble polysaccharide fraction is a low-viscosity emulsifier for acidic beverage emulsions (Yin et al., 2012). Most extracts rich in pectic substances have gelling properties. The gelling mechanism of pectic polysaccharides depends on their degrees of esterification; if the degree of acetyl esterification is low, the extracts are able to gel in the presence of high concentration of sucrose (fractions with more than 50% degree of methyl esterification) or calcium ions (fractions with lesser degrees). Extracts containing pectic substances from cranberry juice are highly viscous and, at low pH, are able to gel at lower sucrose concentrations (38-41%) that pectins from apple and *Citrus* sp. (Baker & Kneeland, 1936). However, sugar beet pectic polysaccharides have many clustered acetyl esterified galacturonic acid residues in their sequence, and therefore do not gel (Harris & Smith, 2006). Nevertheless, they are still used as emulsion stabilizers and to improve the texture of minced meat products. Pectins can also be chemically modified to change their gelling properties; for example, gels made with amidated pectin reversibly dissolve when heated (Vithanage et al., 2010).

2.8.2. Biological properties

The main biological property of non-starch polysaccharides is their total or partial indigestibility. Starch itself can, in several cases, partially resist digestion (resistant starch) (Sajilata et al., 2006).

For this reason, in the past they were considered an irrelevant or even negative component of foods, since their presence reduces the absorption of other nutrients in the intestine. Since the 1950's, the beneficial effects of a regular intake of dietary fiber have been growingly understood (Brownlee, 2011). It is now known that fiber has positive effects on the gastrointestinal motility and on the population of gut microorganisms (microbiota), which ferment the polysaccharides (Tester & Al-Ghazzewi, 2013), and pectic polysaccharides have shown to selectively promote the growth of beneficial gut bacteria (Tingirikari, 2019). In turn, the presence of a healthy microbiota is required for a correct morphology of the intestinal mucosa and the development of gut's immune system (Macpherson & Harris, 2004), two aspects that are compromised in inflammatory bowel disease. The positive correlation between dietary fiber consumption and reduced risk of other diseases -such as type II diabetes, obesity and cardiovascular diseases- which is likely to be microbiota-mediated, is suggested by some population studies (Hu et al., 2001; Ou et al., 2013; Pietinen et al., 1996). For these reasons, the fiber content of several food products is deliberately increased with polysaccharides. While in cereal products the added fiber is composed of resistant starch and β-glucans (Yokoyama et al., 1997), dairy products are often enriched with pectic substances, inulin, guar gum and carboxymethyl-cellulose. The degree of methyl esterification of the pectic polysaccharides may also play a role in their *in vivo* fermentation by gut bacteria, as shown by *in vitro* studies on isolated bacterial strains (Olano-Martin et al., 2002), in which lower esterification corresponded to higher fermentation.

2.9. Oligosaccharides

Oligosaccharides (OS) are oligomers of 3-20 glycosyl residues. Their abundance *in vivo* is much lower than the other carbohydrates, as they mostly exist transitorily as intermediates of polysaccharide synthesis or catabolism. Because of this, most commercial OS are obtained by partial digestion of polysaccharides (xylans, starch, mannans) or glycoproteins with acids or enzymes. Such is the case for xylo-OS (XOS), isomalto-OS (IMOS), malto-OS, cyclodextrins, gentio-OS and mannan-OS (MOS). Galacto-OS (GOS), lactosucrose and glycosyl-sucrose are instead synthetized from disaccharides (lactose or sucrose) by adding glucidic residues to them, using enzymatic activities and chemical condensation (Tanaka et al., 1993). Fructo-OS (FOS) are obtained either by hydrolyzing fructans or by elongating sucrose; these two methods result in slightly different products.

Soybean OS, raffinose and stachyose are the only OS functional ingredients directly produced by purification of a plant extract (Crittenden & Playne, 1996). Industry produces these OS as mixtures with variable degree of polymerization. Depending on the degree of purification, residual monosaccharides and polysaccharides may be also present (S. Kim et al., 2003). Table 2.2 illustrates the source, structure and degree of polymerization of selected OS industrially extracted from plants or produced from plant polysaccharides. Raffinose and stachyose are reported as separate products, but mixtures of the two are also sold as "soybean OS" (Crittenden & Playne, 1996).

2.9.1. Pectic oligosaccharides

Current research is aiming at the conversion of pectic polysaccharides into pectic oligosaccharides (POS) that can be obtained by chemical or enzymatic hydrolysis of pectin-rich products, such as sugar beet (Holck, Hjernø, et al., 2011) and wastes obtained by the processing of olives (Lama-Muñoz et al., 2012), pumpkins (Du et al., 2011), oranges (Martínez Sabajanes et al., 2012) and haws (S. Li et al., 2013). Given the complexity of the starting material, the result of chemical processes is always a mixture of different OS with various degrees of polymerization. For example, while HG is solely converted to oligogalacturonides, RGI yields galacto-OS, arabino-OS and a variety of OS with heterogeneous glycosyl residues (Holck et al., 2014). Treatment of cranberry hull with a commercial mixture of pectin-degrading enzymes was found to release both pectic arabino-OS and xyloglucan fragments (Sun et al., 2015). The structure of these mixtures is generally characterized only in terms of degree of polymerization, percentage of acid and neutral residues. However, better-defined OS can be obtained by hydrolysis catalyzed by pure enzymatic activities (Holck, Hjernø, et al., 2011). The degree of esterification of the OS can be controlled by subjecting the pectic polysaccharides to a demethylation pre-treatment (Combo et al., 2013).

2.9.2. Technological properties

Several OS are used as functional ingredients. Their intermediate molecular weight makes them better viscosity-increasing ingredients than sucrose and better sweeteners than polysaccharides (the sweetness ranging from 0.3 to 0.6 times that of sucrose). The inclusion of IMOS in food products results in a higher retention of water, with moisturizing effects and simultaneously reducing the moisture available to spoiling microorganisms (Nakakuki, 2003).

Table 2.2 Selection of oligosaccharides used as functional ingredients, their structure, source and degree of polymerization (DP) (Crittenden & Playne, 1996; Greffe et al., 2005; Patel & Goyal, 2011; Tanaka et al., 1993).

Source	Oligosaccharide	Structure	DP
Cereal xylans	XOS	$[\beta\text{-}(1\rightarrow4)\text{-D-Xyl}]_n$	2-9
Xyloglucans	Xyloglucan-OS	$[\beta\text{-}(1\rightarrow4)\text{-D-Glc}]_n$ [a]	7-9
Starches	IMOS	$[\alpha\text{-}(1\rightarrow6;1\rightarrow4)\text{-D-Glc}]_n$ [b]	2-5
	Malto-OS	$[\alpha\text{-}(1\rightarrow4)\text{-D-Glc}]_n$	2-7
	Cyclodextrins	$[\text{-}\alpha\text{-}(1\rightarrow4)\text{-D-Glc}]_n$ (cyclical)	6-12
	Gentio-OS	$[\beta\text{-}(1\rightarrow6)\text{-D-Glc}]_n$	2-5
Chicory root fructans	FOS	$[\beta\text{-}(1\rightarrow2)\text{-D-Fru}]_n$; $\alpha\text{-D-Glc-}(1\rightarrow2)\text{-}[\beta\text{-D-Fru-}(1\rightarrow2)]_n$	2-10
Sugar beet arabinan	Arabino-OS	$[\alpha\text{-}(1\rightarrow5)\text{-L-Ara}]_n$	3-8
Soybean	Raffinose	$\alpha\text{-D-Gal-}(1\rightarrow6)\text{-}\alpha\text{-D-Glc-}(1\rightarrow2)\text{-}\beta\text{-D-Fru}$	3
	Stachyose	$\alpha\text{-D-Gal-}(1\rightarrow6)\text{-}\alpha\text{-D-Gal-}(1\rightarrow6)\text{-}\alpha\text{-D-Glc-}(1\rightarrow2)\text{-}\beta\text{-D-Fru}$	4

[a] Xyloglucan-OS have α-(1→6)-D-Xyl and β-D-Gal-(1→2)-α-D-Xyl side chains (Fry et al., 1993);

[b] IMOS have a branched structure featuring an α-(1→6) backbone.

OS find also application as cryoprotectants (Auh et al., 1999) and in controlling the heat-induced modification of proteins and starch (Zhu et al., 2019). Cyclodextrins, thanks to their ring structure, are useful for chelating foul smelling and tasting components and to stabilize reactive compounds, such as vitamins and minerals in food formulations (Nakakuki, 2003). While POS are not currently used to confer technological properties to food, they exhibit viscosity properties similar to the other OS. However, due to their completely different composition, they likely possess unique sweetness profiles. Oligogalacturonides and RGI backbone fragments, being polyanionic, could be used to form biocompatible films by directed self-assembly with positively charged molecules (Combo et al., 2013).

2.9.3. Biological properties

Sucrose-derived OS are not fermentable by mouth microorganisms, making them non-cariogenic sweeteners. In particular, glycosyl-sucrose and the related maltooligosyl-sucroses (degree of polymerization 4-11) reduce the cariogenicity of sucrose by inhibiting specific *Streptococcus mutans* enzymes (Saehu et al., 2013). Like polysaccharides, most of the OS are non-digestible by humans, but fermented by microorganisms in the large intestine. The main difference is that most polysaccharides indiscriminately favor the growth of beneficial (*Bifidobacterium* and *Lactobacillus* ssp.), commensal and potentially pathogenic (e.g. Proteobacteria) microorganisms, while some OS selectively increase the number of beneficial microbes (prebiotic effect). Among these are XOS (Aachary & Prapulla, 2011), GOS (Smiricky-Tjardes et al., 2003), FOS (Howard et al., 1995) and IMOS (Kohmoto et al., 1991). Studies have associated inflammatory bowel disease to variations in the gut microbiota (Gruber & Haller, 2015). Alterations of the microbiota are also related to the occurrence of irritable bowel syndrome, metabolic (including cardiovascular) diseases (Burcelin et al., 2015) and colon cancer (Ou et al., 2013), though their causal relationship is still unclear. POS are known to possess not only prebiotic properties (Chen et al., 2013; Du et al., 2011; Gómez et al., 2014; Olano-Martin et al., 2002) but also antibacterial activity against *Escherichia coli* K-12 (S. Li et al., 2013). POS from orange albedo were shown to reduce, *in vitro*, the invasion of *Campylobacter jejuni* in the Caco-2 epithelium (Ganan et al., 2010).

A phenolic-free, OS-rich extract of cranberry, containing arabino-OS and xyloglucan-derived OS, was found to have an anti-biofilm effect on the *E. coli* strains CFT073 (uropathogenic) and MG1655 (non pathogenic) *in vitro* (Sun et al., 2015).

2.10. Isolation of polysaccharides from plant cell wall

In industrial settings, pectic polysaccharides are commonly recovered from plant material using hot acid extraction (Liu et al., 2010). At laboratory scale, the method has been successfully enhanced with the application of microwaves and ultrasounds. Alternative methods have been developed, such as employing enzymes (Matora et al., 1995), compressed hot water (Muñoz-Almagro et al., 2019; Wang & Lü, 2014), alkali (Ji et al., 2018; Zykwinska et al., 2006), or simply by fine wet grinding and sedimentation (Fujio & Furuta, 1989). Hemicelluloses are extractable by alkali from plant sources in which they are abundant such as sugarcane (Jayapal et al., 2013). For analytical and research purposes, it is often a necessity to separate the plant cell wall in several fractions, each containing polysaccharides with specific, non-overlapping properties. A frequently used approach to achieve this is the sequential extraction with different aqueous solvents.

2.10.1. Sequential extraction

Several alternative designs of sequential extraction of polysaccharides exist. In all cases, the sequence of solvents and conditions applied to the plant material is increasingly harsh, as to first recover the least strongly interconnected polymers, and progressively the ones that are more integrated in the cell wall matrix. Often, the initial dry plant material is pre-treated by suspending it in 70% ethanol and removing the supernatant, that contains the smaller compounds from cell wall. The precipitate, enriched in cell wall polysaccharides, is referred to as alcohol insoluble solids (AIS). A commonly used sequence of solvents is hot weakly acidic buffer or hot water, followed by hot chelating agents, followed by diluted and then concentrated alkali and/or acids (Coimbra et al., 1994; Hilz et al., 2005; Prabasari et al., 2011; Rozi et al., 2019; Sengkhamparn et al., 2009; Vierhuis et al., 2000). Hot buffer and chelating agent extracts contain pectin and pectic acids, respectively. Diluted alkali extracts contain the less strongly bound hemicelluloses and of protopectin, while concentrated alkali extract the more tightly bound ones. These solvents cannot extract cellulose, which accumulates as a final residue.

2.10.2. Hot acid extraction of pectin

In the industrial process, the plant material is extracted once or twice with a mineral acid solution at pH 2 and 70-90 °C. The extraction time varies according to the starting material and the desired quality of the extracted pectin (longer times cause greater de-esterification). The solution is then separated by filtration, and pectin is purified by precipitation with ethanol, isopropanol or aluminum chloride (Joye & Luzio, 2000). In small scale experiments, different conditions are usually explored in order to optimize the extraction of specific polysaccharides from a given source (Liu et al., 2010).

2.10.3. Microwave-assisted process

Exposing a homogenous water-based mixture to microwaves allows for a rapid and even heating, which accelerates acid and alkaline hydrolysis reactions. Various setups using this technique for pectin extraction have been tested on various plant materials. For example, orange albedo was suspended in hydrochloric acid, directly heated with microwave and filtered, repeating the process up to three times (Fishman et al., 1999); pumpkin powder was first treated for 1 hour with acid, then heated one single time with microwave and filtered (Yoo et al., 2012). It is also possible, by modulating the reaction conditions, to obtain extracts enriched with specific polysaccharides; such an approach was used in the extraction of RGI-rich fractions from potato cell wall material. Potato was suspended in potassium hydroxide, heated with microwaves, filtered and dialyzed (Khodaei et al., 2016).

2.10.4. Ultrasound-assisted process

The use of ultrasounds to generate localized cavitation in the water- or solvent-swollen plant material, thus breaking the cell wall and facilitating the solubilization of polysaccharides, has been explored since the ‘90s (Ebringerová & Hromádková, 2010). Recent examples include the extraction of polysaccharides from mulberry leaves (Ying et al., 2011), litchi seeds (Chen et al., 2011) and seaweeds (Alboofetileh et al., 2019; Vázquez-Rodríguez et al., 2020).

2.10.5. Enzymatic extraction of pectic polysaccharides

Among the many cell wall-degrading enzymes, the ones that can be used for the recovery of polysaccharides are those catalyzing their partial hydrolysis, thus releasing them from the matrix.

Examples are cellulase (EC 3.2.1.4), hemicellulose-degrading enzymes (mannan endo-1,4-β-mannosidase EC 3.2.1.78; endo-1,4-β-xylanase EC 3.2.1.8), HG-degrading enzymes (polygalacturonase EC 3.2.1.15; pectate lyase EC 4.2.2.2; pectin lyase EC 4.2.2.10) and RG I-degrading enzymes (rhamnogalacturonan hydrolase EC 3.2.1.171; endo-α-1,5-L-arabinanase EC 3.2.1.99; arabinogalactan endo-β-1,4-D-galactanase EC 3.2.1.89; endo-β-1,3-D-galactanase EC 3.2.1.181). If the target of the extraction is to recover pectic polysaccharides, enzymes can be used for the hydrolysis of protopectin leading to the generation of water-soluble pectin. For example, the extraction of pectin from lemon pomace was carried out using endo-polygalacturonase from *Aspergillus niger* (Contreras-Esquivel et al., 2006). However, the presence of non-pectic polysaccharides may interfere with the extraction both by posing a physical limitation to the enzymatic activity and by being bound to the otherwise soluble fragments; one approach to overcome this issue is to hydrolyze the non-pectic polysaccharides as well. Viscozyme L, a commercial enzyme mixture, which contains pectin-degrading and β-glucosidase activities, was used to recover pectin from *Citrus junos* (Lim et al., 2012). Pectin was also extracted from pumpkin and sugar beet pulp using the supernatant of a non-pathogenic *Bacillus polymyxa* culture, whose growth conditions were selected in order to minimize the secretion of pectin-degrading enzymes (Matora et al., 1995). Lastly, it is possible to solely focus on the degradation of the non-pectic components of the cell wall, successively recovering pectic substances by alcohol precipitation. A pectin yield of 6.85% from rapeseed (*Brassica napus*) press cake was obtained, using a one-pot hydrolysis with fungal cellulase and *Bacillus licheniformis* protease (Jeong et al., 2014).

2.11. Generation of oligosaccharides from plant cell wall

Soybean OS can be directly isolated from the by-products of the preparation of protein concentrates (Crittenden & Playne, 1996), soymilk (Wu et al., 2012) or other soy products (Wang et al., 2012). They are generally purified by centrifugation, acid extraction and ultrafiltration, though the conditions can vary depending on the prior passages that the raw beans undergo. The other OS obtained from plant cell walls are derived from polysaccharides *via* enzymatic or chemical hydrolysis, followed in some cases by enzymatic transglycosylation.

2.11.1. Enzymatic approaches

One way to produce commercial FOS is by the endo-hydrolysis of inulin, a storage polysaccharide of which chicory is rich, by inulinase (EC 3.2.1.7). Another method for the production of commercial FOS with similar results involves the use of the chicory enzyme fructan:fructan fructosyl transferase (EC 2.4.1.100), which transfers fructose residues from inulin to a starting fructose solution (Van den Ende et al., 1996). Malto-OS are obtained from starch by the combined action of a debranching enzyme (isoamylase EC 3.2.1.68 or pullulanase EC 3.2.1.41) and α-amylase (EC 3.2.1.1). Maltoolygosyl-sucroses, malto-OS with a terminal fructose, can be produced by adding sucrose to the partly hydrolyzed starch using transglycosylating enzymes (Saehu et al., 2013). In the industrial production of isomalto-OS, starch is sequentially depolymerized to maltose by α-amylase and β-amylase (EC 3.2.1.2). Then, maltose becomes the donor and acceptor of glucose residues for the transglycosylation activity of α-glucosidase (EC 3.2.1.20). An alternative last step is using dextransucrase (EC 2.4.1.5) from *Leuconostoc mesenteroides*, which requires the addition of sucrose as glucose donor (Rabelo et al., 2009). Cyclodextrins are produced from starch by controlled hydrolysis with debranching enzymes, followed by the enzymatic cyclization performed by bacterial cyclomaltodextrin glucanotransferase (EC 2.4.1.19) from the genus *Bacillus* (Biwer et al., 2002; Davis & Higson, 2011). All the orthologs produce cyclodextrins with a range of degrees of polymerization, the most common ones being 6, 7 and 8. The conversion of xylans to XOS is achieved with endo-1,4-β-xylanase (Akpinar et al., 2007; Jayapal et al., 2013).

POS can be obtained by treating plant cell wall materials rich in pectic polysaccharides, such as orange peel wastes (Li et al., 2016), with multi-enzymatic biocatalysts, including pectinases, cellulase and hemicellulases. It is also possible through the sequential application of specific enzymes to obtain well-defined POS from AIS. The following sequence [pectin lyase (EC 4.2.2.10) → RG I-debranching enzymes (β-galactosidase, EC 3.2.1.23; endo-1,4-β-galactanase, EC 3.2.1.89; endo-α-arabinanase EC 3.2.1.99; α-arabinofuranosidase EC 3.2.1.55) → rhamnogalacturonase I lyase, EC 4.2.2.23)], with each step followed by membrane filtration, was used by Holck et al. (2011) to recover the OS.

2.11.2. Non-enzymatic processes

The xylans found in ligno-cellulosic materials can be degraded to soluble XOS by treatment with acidic hot water or steam (auto-hydrolysis) without affecting the other cell wall constituents (Nabarlatz et al., 2005; Reis et al., 2003; Samanta et al., 2012). Xyloglucan-OS can be obtained by exposing xyloglucan to sonication (Vodenicarova et al., 2006).

2.12. Effects of enzymatic reaction parameters on oligosaccharide generation

In general, the enzymatic conversion of cell wall materials to OS is limited by their scarce solubility. Temperature, pH, enzyme and substrate concentration, as well as reaction time also influence the reaction outcome in terms of yield and characteristics of the products.

In a work on the batch production of XOS by endoxylanase from *Aspergillus oryzae*, it was found that at concentrations greater than 6% (w/v), alkali-pretreated corn cob was partly insoluble, but optimization of the reaction parameters allowed for high yields (81%) (Aachary & Prapulla, 2009).

A potential solution to the solubility problem is adsorbing the enzyme on the substrate. This approach was attempted by incubating mixtures with varied amounts of bacterial xylanase and wheat straw at 4 °C (Zilliox & Debeire, 1998). Only a small fraction of the enzyme leached during the first of the five hours of reaction at 60 °C, and the highest yield (11%) was achieved with the use of 300 μg of enzyme per g of straw.

2.13. Strategies for the esterification of poly- and oligosaccharides

The physicochemical and biological properties of polysaccharides and OS can be modified to increase the range of their applications. Common approaches include the amidation of pectin, obtained by the reaction of ammonia with the carboxylic acids of galacturonic acid residues, that can reduce its solubility (Reitsma et al., 1986; Vithanage et al., 2010). Cellulose esters, such as acetate and nitrate, have improved solubility in water and find applications in the synthesis of membranes and films (Voicu et al., 2016). Esterified polysaccharides also occur in nature; in addition to the methyl and acetyl esterification of pectic polysaccharides mentioned in paragraph 2.3, esterification with ferulic acid, a phenolic compound, is also found in the arabinoxylans of cereals and in the neutral side chains of the pectic polysaccharides of plants (Ralet et al., 1994).

Some of these feruloylated residues were identified as 5-O-feruloyl-L-arabinose, 2-O-feruloyl-L-arabinose and 6-O-feruloyl-D-galactose (Colquhoun et al., 1994).

2.13.1. Feruloylation

The esterification of polysaccharides and OS can be achieved by the chemical synthesis and by the enzymatic catalysis. An example of chemically feruloylated polysaccharide is starch, which after reaction with ferulic acid chloride in acid dimethyl sulfoxide showed an increase in water-holding, reduced viscosity and improved resistance to retrogradation (Ou et al., 2001). Enzymatic synthesis of feruloylated mono- and oligosaccharides has been reported. A ferulic acid transferase and an arabinoxylan trisaccharide extracted from *Oryza sativa* were mixed with chemically synthesized feruloyl-CoA to obtain the ester (Yoshida-Shimokawa et al., 2001) in a reaction performed at 30 °C for 12 h in phosphate buffer at pH of 6. The transesterification activity of type C feruloyl esterase from *Myceliophthora thermophila* was used to convert methyl ferulate into 5-O-feruloyl-L-arabinose in presence of a microemulsion of aqueous buffer in organic solvent, prepared by mixing of n-hexane, t-butanol and buffer piperazine-hydrochloride (20 mM, pH of 6) in various proportions (Topakas et al., 2005). The same approach was applied to obtaining arabinose disaccharides and short OS mono-feruloylated at the nonreducing end (Vafiadi et al., 2007). In acetonitrile and methyl isobutyl ketone, the commercial enzyme mixture Lipolase 100T showed transesterification activity that was employed to feruloylate methyl-α-L-arabinofuranose and methyl-β-D-glucopyranose, using vinyl ferulate as donor (Mastihubová et al., 2006). Recently, feruloyl esterase from *Humicola insolens*, immobilized on various supports, was successfully utilized for the feruloylation of raffinose and XOS in microemulsions of water in n-hexane and 2-butanone (Tamayo-Cabezas & Karboune, 2019). In the tested conditions, it was found that the type of support affected the esterification activity of the enzyme, and porous epoxy-activated supports modified with iminodiacetic acid were found to allow good immobilization yield and enzymatic activity.

2.13.2. Esterification with fatty acids

The esterification of oligosaccharides with fatty acids results in amphiphilic molecules that have found applications as surfactant ingredients in the formulation of food, drugs and cosmetic products (Allen & Tao, 1999).

Some of these compounds also show antibacterial properties (Devulapalle et al., 2004; Smith et al., 2008). Fatty acid esters of carbohydrates can be produced with a variety of chemical methods, such as condensation in pyridine, used to esterify sucrose (Hass, 1968), or protective group chemistry (Smith et al., 2008). In organic solvents, several bacterial lipases and proteases thermolysin and subtilisin Carlsberg (Lee et al., 2007; Pérez-Victoria & Morales, 2006, 2007; Plou et al., 2002) display a transesterification activity that has been employed to obtain acylated di- and trisaccharides. In some cases, a high regioselectivity is also observed.

2.14. Structural characterization of polysaccharides and oligosaccharides

The structure of polymeric sugars has great variability among species, tissues and growth stages. Cell wall extracts contain several different poly- and oligosaccharides. The determination of the overall composition (neutral sugar residues, uronic acid residues, degrees of acetyl and methyl esterification) of such mixtures is commonly performed with colorimetric and chromatographic methods (Hilz et al., 2005; Vierhuis et al., 2000). However, to identify a poly- or oligosaccharide, the sequence of its residues and the type of glycosidic bonds must be identified. The experimental approach to do so consists in the further purification of the cell wall extracts, with chromatography or further extraction, followed by the controlled partial digestion and chromatographic separation of the fragments (Hilz et al., 2007; Reis et al., 2003). The OS-containing fractions can then be analyzed with mass spectroscopy (MS) and nuclear magnetic resonance (NMR).

2.14.1. High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) methods allow for the simultaneous characterization of a polysaccharide mixture and its separation in fractions with similar molecular size, polarity or partition coefficient, depending on the type of resin used. For example, uronic acid-containing carbohydrates can be separated with anion exchange columns (Khodaei & Karboune, 2013; Massiot, 1994). The High-Performance Anion Exchange Chromatography (HPAEC) setup consists of an anion exchange column in which the polysaccharide or OS mixture is loaded under strong alkaline conditions, so that all the monosaccharidic residues are ionized. The column is then eluted under high pressure with a sodium hydroxide gradient, separating the ionized sugars based on their charge.

HPAEC is usually coupled with a pulsed amperometric detector (PAD) (Coulier et al., 2013; Mazumder et al., 2005; Vierhuis et al., 2001), which measures the concentration of sugars as function of the current produced by their electrochemical re-oxidation to the neutral form. This system is suited for the detection of carbohydrates at very low concentrations. Columns packed with polymethacrylate beads are used for the size exclusion separation of water-soluble polysaccharides and OS (Deery et al., 2001; Saehu et al., 2013; Vandevelde & Fenyo, 1985); since carbohydrates possess low light absorbance, their detection in the eluate is commonly performed by refractive index (RI) detector.

2.14.2. Mass spectrometry

Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two most useful ionization techniques for the characterization of polymeric sugars by MS, because they do not require vaporization of the analyzed compounds and allow the formation of large molecular ions (Garozzo et al., 1999; Mazumder et al., 2005; Peng et al., 2012; Reis et al., 2003). These techniques are however only applicable to OS and short polysaccharides, therefore longer chains must be chemically or enzymatically hydrolyzed to OS fragments. Tandem mass spectrometry approaches, thanks to the fragmentation of the ions (generally achieved by collision-induced dissociation), can be used to determine the structure of the OS and the presence of acetylation (Hilz et al., 2007).

An alternative approach for structure determination is derivatizing the free hydroxylic groups of the OS to methyl esters, hydrolyzing to monosaccharides and further protecting the newly exposed hydroxyl groups with 1-phenyl-3-methyl-5-pyrazolone (PMP). The obtained monosaccharides can then be detected by liquid chromatography-MS to reconstruct the presence of the different linkages from the amount and position of the derivatizations (Galermo et al., 2018; Xu et al., 2018).

2.14.3. Nuclear Magnetic Resonance

Plant cell wall polysaccharides and the derived OS are almost exclusively composed of carbon, hydrogen and oxygen atoms. Therefore, the NMR spectra of ^{1}H and ^{13}C of a pure compound can theoretically provide enough information to elucidate its structure, including the anomeric configuration of the glycosidic bonds and the sequence of glycosyl residues (Rana et al., 2009).

However, in the normal range of energy of NMR spectroscopy, the peaks from repeated residues overlap, introducing uncertainty in the interpretation of the result (Duus et al., 2000). To overcome this limitation, 2D NMR techniques, such as correlation spectroscopy (Sims et al., 1997) and Nuclear Overhauser Effect spectroscopy (NOESY) have been employed. Homonuclear correlation spectra (COSY and TOCSY) favor the complete assignment of the ^{1}H-NMR peaks by showing the presence of coupled atoms as easily distinguishable peaks in a 2D chart, rather than as multiple peaks. Similarly, heteronuclear single-quantum correlation spectra (HSQC) clarify the ^{13}C-NMR peaks. Long-range correlation techniques, such as NOESY and heteronuclear multiple-bond correlation (HMBC), are useful in determining the sequence of glycosyl residues (Chandra et al., 2009; B. Li et al., 2013).

2.15. Evaluation of prebiotic effect

The determination of the fermentation that a polysaccharide or OS undergoes in the human gut is challenging for two reasons. Firstly, *in vivo* experiments are invasive, and thus are performed on model animals (Smiricky-Tjardes et al., 2003), giving results that are not applicable to humans. Secondly, the *in vitro* alternatives, which are much easier to perform, cannot screen the fermentation capacity of the whole microbiota because many of the microorganisms that compose it are not cultivable. *In vitro* experiments also completely ignore the modulation of bacterial activity that results from the interactions between gut tissues and microbiota. Nevertheless, in many cases the assays for prebiotic activity rely on *in vitro* observations. In addition to these experiments, the carbohydrate can be exposed to artificial human gastric juice and human hydrolytic enzymes to study its eventual modifications during its transit in the digestive system (Du et al., 2011).

2.15.1. Fermentation by isolated strains

In this setup, the prebiotic properties of the non-digestible carbohydrate are assessed as differential fermentation by beneficial, commensal or pathogenic monoclonal strains of gut microorganisms (Van Laere et al., 2000). Alternatively, the fermentation of the carbohydrate and of known prebiotics by one (Du et al., 2011) or more (Bao Yang et al., 2011) beneficial bacterial strains can be compared.

In general, the selected strains are grown in anaerobic conditions at 37 °C to simulate the human intestine, in a sugar-free liquid terrain to which the carbohydrate whose prebiotic properties are to be evaluated is added as the sole carbon source. The fermentation can be monitored either by bacterial growth, that can be measured by colony count on selective solid media or by optical density of the liquid culture, or by the quantification of selected fermentation by-products, such as short chain fatty acids (Manderson et al., 2005).

2.15.2. Fermentation by fecal inoculum

These experiments aim at a better representation of the gut microbiota with its interspecific interactions, by incubating human fecal matter in a batch fermenter with rich growth medium under anaerobic conditions. While the fecal inoculum is unlikely to possess all the microbial strains found in the gut, it can be expected to contain the beneficial *Bifidobacteria* and *Lactobacilli*, and the commensal *Clostridia* and *Bacteroides* (J. Chen et al., 2013). At given times, aliquots of the fermentation medium are taken and analyzed. The colony forming units of bacteria from the cited genera are counted by plating them on selective solid media, so to assess whether the added compound selectively favors the growth of beneficial strains. The short-chain fatty acids (SCFA), such as butyric acid, propionic acid and caproic acid, are the main products of bacterial fermentation. Their presence and ratio can be measured by gas chromatography (Rehman et al., 2008) and changes in their composition are an indicator of alterations in the microbial population, some of which are impossible to detect by plating.

CONNECTING STATEMENT 1

In the literature review, presented in Chapter II, the structural features and functional properties of cell wall polysaccharides and oligosaccharides are discussed. The characterization of cranberry pomace composition and its cell wall material, together with the assessment of two approaches for the extraction of oligosaccharide from it, are reported in Chapter III. Proximate analysis and dietary fiber quantification were performed on cranberry pomace by-product from industrial juice production. The monosaccharide profile of the fiber, which consisted primarily of carbohydrates, was determined by high performance anion exchange chromatography. Microwave-assisted alkaline extraction and treatment with commercial pure and multi-enzymatic hydrolases were applied to the pomace and the extracted oligosaccharides were characterized in terms of molecular weight and composition. The effect of different pomace particle sizes on the yield and composition of the extract generated by the multi-enzymatic catalyst Depol™ 740L were compared.

The results from this study were presented at the 2016 IFT Annual Meeting & Food Expo-The Institute of Food Technologists and submitted to a scientific journal.

Spadoni Andreani, E. & Karboune, S. (2020). Comparison of Enzymatic and Microwave-Assisted Alkaline Extraction Approaches for the Generation of Oligosaccharides from American Cranberry (*Vaccinium macrocarpon*) Pomace. Journal of Food Science 85, 2443-2451.

CHAPTER III. COMPARISON OF ENZYMATIC AND MICROWAVE-ASSISTED ALKALINE EXTRACTION APPROACHES FOR THE GENERATION OF OLIGOSACCHARIDES FROM AMERICAN CRANBERRY (*VACCINIUM MACROCARPON*) POMACE

3.1. Abstract

Cranberry pomace obtained from industrial juice production was characterized by proximate composition analysis and monosaccharide profile of the dietary fiber. Extraction of carbohydrates from pomace was investigated using microwave-assisted alkaline method and five commercial biocatalysts (pure *endo*-galactanase and four multienzyme biocatalysts). The extracts obtained from microwave-assisted approach had average total sugars yield of 21.3% and contained mostly oligosaccharides in the degree of polymerization range of 7-10. All multienzyme biocatalysts led to yields similar or higher than microwave-assisted approach (23.4-42.0%), but mainly generated shorter oligosaccharides with a degree of polymerization of 2 to 5. Compared to cranberry pomace dietary fiber, microwave-assisted extracts were enriched in pectic oligosaccharides, while the enzymatic extracts were enriched in glucans and had less rhamnose and galactose. Pomace ground for 5 min or more by ball mill assumed a powdery consistence. Longer milling didn't affect particle size but increased their roughness. Such physical changes had no effect on the efficiency of multi-enzymatic treatment.

3.2. Introduction

The physical and biological properties of naturally occurring plant cell wall polysaccharides and their corresponding oligosaccharides have been of a great interest, and many of them find application as functional food ingredients. For instance, pectic polysaccharides are widely used as gelling agents and emulsion stabilizers (Jiang & Du, 2017; A. G. J. Voragen et al., 2009), and can be added in foods as a non-digestible fraction (dietary fiber) to increase the satiating effect with a minimal contribution to the calorie count. A diet rich in fiber has also been positively correlated to gastro-intestinal health (Brownlee, 2011; Cui et al., 2019). Furthermore, non-digestible oligosaccharides as prebiotics can promote the intestinal health by stimulating the growth of beneficial bacteria in the gut (Smiricky-Tjardes et al., 2003), reducing the risk of colon cancer (Olano-Martin et al., 2003) and inhibiting the invasion of pathogenic bacteria in intestinal epithelium models (Ganan et al., 2010; E. Li et al., 2019), while being low-cariogenic sweeteners (Saehu et al., 2013), and promoters of mineral absorption (Cashman, 2006). Consequently, there is a great interest in the generation of non-digestible oligosaccharides from new sources with improved prebiotic activity and other physiological properties.

Pectic polysaccharides have been identified as potential sources for the production of selected prebiotics, such as galactooligosaccharides, arabinooligosaccharides and galacturonoligosaccharides (Al-Tamimi et al., 2006; Di et al., 2017; Olano-Martin et al., 2002; Silk et al., 2009; Vigsnæs et al., 2011).

Conventionally, pectic polysaccharides are isolated from food processing by-products, whose main fraction is composed of plant cell wall (Heredia et al., 1995). Despite the fact that pectic polysaccharides are present in the majority of plant cells, apple pomace and citrus fruit peel are the main sources used for their extraction at commercial scale (P. Srivastava & Malviya, 2011). Other plant cell-rich by-products are used as animal feed, incinerated or directly disposed in landfills. There is an increasing demand for the effective use of the plant cell-rich by-products as a source of functional ingredients. This is the perspective in which previous works from our research group were conducted on potato cell wall (Khodaei & Karboune, 2013), using the microwave-assisted and the enzymatic extraction approaches.

Cranberry (*Vaccinium macrocarpon*) is a small perennial shrub of the family Ericaceae endemic to temperate areas in the east of North America, where nearly 90% of the cultivated plants is currently grown. The edible fruits, produced annually, are red ovoid berries with a bitter and astringent taste. Cranberry global annual production exceeds that of blueberry (Food and Agriculture Organization of the United Nations 2017), and 95% of the harvested fruits are processed into jams, sauces and juice (USDA - National Agricultural Statistics Service, 2017). Cranberry juice and blends containing it are popular beverages consumed worldwide, especially after the rise in popularity of the fruit as health-promoting food (Hancock et al., 2007). A number of studies have characterized the cell wall components of cranberry and *Vaccinium* genus fruits (Deng et al., 2013; Fan et al., 2010; Gouw et al., 2017; Hilz et al., 2005; Park & Zhao, 2006; Ross et al., 2015), revealing the presence of pectic polysaccharides in amounts comparable to those present in apple pulp (10-15% of dry weight) (Rolin, 1993). Despite the fact that the cranberry pomace is rich in plant cell polysaccharides, it has been mainly explored as a source of phenolic antioxidants (Jara-Palacios et al., 2019). Indeed, the health-promoting effects of the cranberry were the subjects of extensive studies focused on its phenolic constituents (Caillet et al., 2012, 2011; Lacombe et al., 2010; Parry et al., 2006).

However, Sun et al. (2015) have demonstrated that in addition to the anti-infective properties of cranberry proanthocyanidins and other polyphenols, oligosaccharide components in cranberry exhibited an anti-biofilm effect against *Escherichia coli* biofilm. Owing to the fact that conventional extraction can lead to the use of large amount of solvents, energy, time and generation of carbon footprints, there has been an increased focus on green technologies for extraction of functional ingredients (Khodaei et al., 2016; Mtetwa et al., 2020; Petkova et al., 2017). The present work is aimed at the extraction of cell wall carbohydrates (e.g. pectic oligo/polysaccharides) from cranberry pomace obtained from industrial juice production using the microwave-assisted alkaline extraction and the enzymatic approaches. Two power levels and two alkali molarities were tested for microwave-assisted extraction, while five different biocatalysts were tested for the enzymatic extraction. The effects of these extraction techniques on the yield and the characteristics of pectic compounds were discussed.

3.3. Materials and methods

3.3.1. Materials

Cranberry pomace was provided by Atoka Cranberries Inc., Manseau, QC, Canada and stored at -20 °C. Depol™ 740L and 670L were provided from Biocatalysts Ltd., Cardiff, UK. Pectic arabinan and *endo*-galactanase were purchased from Megazyme Ltd., Bray, Ireland. Viscozyme® L, Pectinex® Ultra SPL, *endo*-polygalacturonase, and the analytical grade reagents were from Sigma-Aldrich Co., St. Louis, MO, USA.

3.3.2. Preparation of cranberry pomace

Cranberry pomace was blended for 40 seconds with a Model 7011C commercial blender (Conair corporation, Stamford, CT, USA) and transferred into a crystallizing dish. Pomace was dried for 18 h at 70 °C in vacuum oven. Blended cranberry pomace was obtained by blending again the dried pomace. Sieved cranberry pomace was obtained by cycles of blending for 40 seconds followed by sieving through a #16 mesh (1.18 mm) sieve. Milled cranberry pomace was obtained from dried pomace by milling with a MM400 steel ball mixer mill (Retsch GmbH, Haan, Germany). At each cycle, the miller was loaded with 0.5 g of pomace and operated at 28 Hz. Multiple cycles were run in order to prepare the needed amounts of sieved and of milled pomace. The obtained pomaces were stored at 4 °C in sealed bottles.

3.3.3. Scanning electron microscopy analysis

The particle size and microscopic structure of blended cranberry pomace and of milled cranberry pomace obtained upon four milling times (1, 5, 15 and 30 min) were characterized with a TM3000 Benchtop scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

3.3.4. Proximate composition analysis of cranberry pomace

Moisture content of cranberry pomace was determined with a variation of AOAC method 927.05 by drying for 18 h at 70 °C in vacuum oven. Total fat content of sieved cranberry pomace was determined using a modified AOAC method 945.16 (Soxhlet extraction of 1 g with 150 ml petroleum ether for 6 h). Ash content was measured by AOAC method 942.05, charring 2 g of sieved cranberry pomace for 30 min, followed by incineration in muffle furnace (5 h at 525 °C). Crude protein content was determined on 0.1 g of sieved cranberry pomace by Dumas method (Saint-Denis & Goupy, 2004) with a FP 628 analyser (LECO Corporation, St Joseph, MI, USA). Dietary fiber content was determined on 1 g of sieved cranberry pomace using the total dietary fiber assay kit (Sigma-Aldrich Co., USA) with a variation of the AOAC method 985.29 (Latimer, 2016), in which α-amylase, protease and amyloglucosidase activities were used to digest the starting material. The remaining fiber was precipitated with ethanol, washed with ethanol and acetone, filtered and, once dry, its protein and ash content were determined and subtracted from the dry weight. All analyses were performed in triplicate. Part of the dry fiber residue recovered after filtration was stored at room temperature in desiccator.

3.3.5. Microwave-assisted extraction

A sample of sieved cranberry pomace was placed in a Pyrex extraction chamber and suspended in KOH (0.25 M or 0.5 M) solution to reach a ratio of 1:90 (w/v). The microwave-assisted alkaline extraction was performed at 85 W/g or 242 W/g for 8 min with a Synthewave 402 microwave reactor (Prolabo Co., Paris, France). Immediately after the irradiation the supernatants were recovered by vacuum filtration on Whatman® 47 mm GF/D glass microfiber (GE Healthcare Life Sciences, Marlborough, MA, USA) and brought to pH 7 from their alkaline pH (13.3 and 13.6 for respectively 0.25 M and 0.5 M KOH) with approximately 6 ml of 1.2 M HCl. Equal volumes of extracts obtained by microwave-assisted alkaline extraction and anhydrous ethanol were mixed.

After 45 h, the mixtures were centrifuged for 25 min at 20,000 ×g with an Avanti J-25I centrifuge (Beckman Coulter Inc., Brea, CA, USA) at 4 °C. The precipitates, representing oligo- and polysaccharides, were collected and freeze-dried.

3.3.6. Enzymatic extraction

Enzymatic extractions were performed using commercial enzyme endo-galactanase and multi-enzymatic biocatalysts (Viscozyme® L, Depol™ 740L, Depol™ 670L and Pectinex® Ultra SPL). The enzymes were dissolved in sodium acetate buffer (10 mM; pH 5.5) to yield 2 U of enzymatic activity per ml of reaction (for the multi-enzymatic biocatalysts, 2 U/ml of arabinanase activity, determined on pectic arabinan by DNS reducing sugar method). Then, 5% w/v of sieved cranberry pomace was added to the solutions in order to yield a ratio of 40 U per gram of pomace. The reaction mixtures were incubated for 48 h at 35 °C under shaking at 200 rpm in an Excella E24 incubator (New Brunswick Scientific, Edison, NJ, USA). The reactions with Viscozyme® L, Depol™ 740L and Pectinex® Ultra SPL were monitored at selected times (0, 4, 8, 14, 24, 36 and 48 h) by taking 2 ml aliquots. Enzymes were deactivated by boiling in water bath for 5 min. The reaction mixtures were then centrifuged for 10 min at 14500 rpm in a Minispin Plus microcentrifuge (Eppendorf AG, Hamburg, Germany) to recover the supernatants, that were stored at -80 °C for the analysis.

3.3.7. Effect of pomace morphology on enzymatic extraction

To assess the effect of pomace morphology, the cranberry pomace was investigated as blended, sieved and milled. The enzymatic extraction was initiated by adding 5.5% (w/v) of cranberry pomace to a solution of Depol™ 740L solution (1.1 U/ml reaction) in sodium acetate buffer (10 mM; pH 5.5). The mixtures were incubated at 35 °C for 4.5 h, under an agitation of 200 rpm in an Excella E24 incubator (New Brunswick Scientific, USA). Reactions were stopped by boiling in water bath for 5 min. Supernatants were collected after centrifugation for 10 min at 14500 rpm. Enzymatic extractions were performed in triplicate.

3.3.8. Determination of total neutral sugars and uronic acid contents

Uronic acid content was measured by sulphamate/m-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973). The phenol–sulphuric acid colorimetric assay was used for the determination of neutral sugar content (DuBois et al., 1956).

3.3.9. Monosaccharide profile

Sieved cranberry pomace, cranberry pomace fiber, the recovered extracts and precipitates were subjected to acid hydrolysis according to the method of Khodaei and Karboune (2013). Briefly, 1 ml of a mixture of 37% HCl/methanol (1:4 v/v) was added to the sample (200 µl of liquid extract, or 5% (w/v) of solid in water). The mixtures were incubated at 70 °C for 24 h and dried by heating in water bath. To complete hydrolysis, 3 ml of water and 1 ml of trifluoroacetic acid were added and heated at 100 °C for 1 h. After evaporating the trifluoroacetic acid the samples were neutralized with NaOH and centrifuged at 8,000 rpm for 5 min. The supernatants (0.02 ml) were analyzed by high performance anion exchange chromatography (HPAEC) on a Dionex ICS 3000 system equipped with pulsed amperometric detection using a Carbopac PA-20 column (Dionex Co., Sunnyvale, CA, USA) at a temperature of 30 ºC. Mobile phase was 5 mM NaOH at flow rate of 0.4 ml/min. Rhamnose, arabinose, glucose, xylose, galactose, and mannose were used at varying concentrations as standards.

3.3.10. Molecular weight distribution

A high-performance size-exclusion chromatography (HPSEC) system (Model 1525 binary HPLC pump, equipped with a Model 2414 refractive index detector, Waters Co., Milford, MA, USA) was used to estimate the molecular weight distribution of the carbohydrates. For polysaccharides, a TSK G5000 PWXL (Tosoh Co., Yamaguchi, Japan) column was used, with dextrans (50–670 kDa) and soybean rhamnogalacturonan (0.125-1 g/l) standards for molecular weight and concentration calibration. For oligosaccharides, a TSK G-Oligo-PW column was used with sucrose, kestose, nystose, fructosylnystose and inulin as standards. For both size exclusion chromatography tests, the temperature of the system was 30 °C, the eluent was 0.1 M NaCl, and the flow rate was set at 0.4 ml/min.

3.3.11. Statistical analysis

Statistical analyses were performed using XLSTAT software (Addinsoft, New York, NY, USA) in Microsoft Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test were performed to detect significant differences ($P < 0.05$).

3.4. Results and discussion

3.4.1. Characterization of cranberry pomace

The composition of cranberry pomace and of its dietary fiber fraction was investigated. The results of proximate composition analysis of pomace, shown in Table 3.1, indicate that dietary fiber represents 63% of the dry weight. The dietary fiber content of cranberry pomace reported in the literature covers a wide range varying from 58.7 to 71.2% (Gouw et al., 2017; White et al., 2010b). This high variability may be attributed to the difference in the cultivar, ripeness and industrial processing conditions. The measured ash, crude protein and carbohydrate contents are in good agreement with the values obtained by Park and Zhao (2006); however, these authors reported a low fat content (1.2%, w/w dry weight). The monosaccharide profile of the recovered cranberry dietary fiber fraction is reported in Table 3.1. The results indicate that the polysaccharides contained in cranberry dietary fibers are composed by 60.4% (mol/mol) neutral sugar and 39.5% (mol/mol) uronic acid residues. Uronic acids residues are mainly derived from pectic polysaccharides, where they are originated from homogalacturonan (HG) and the backbone of rhamnogalacturonan I (RG I), which can be ramified with galactan and arabinan side chains (Scheller et al., 2006). The low ratio of glucose compared to other monosaccharides indicates that pectic polysaccharides and hemicelluloses are predominant in cranberry pomace over cellulose. Compared to the cranberry pomace, the monosaccharide profile of bilberry and black currant pomaces (Aura et al., 2015; Hilz et al., 2005) were reported to be characterized by low arabinose (4.9-6%), galactose (5.4- 5.0%) and uronic acids (11.1- 20%) contents, while their glucose, xylose and mannose contents were higher. These last monosaccharides are mostly derived from cellulose and hemicelluloses.

Table 3.1 Chemical composition of cranberry pomace and monosaccharide profile of cranberry dietary fiber.

Parameter	
Moisture	55.1 ± 0.3%
Total fat [a]	6.3 ± 0.6%
Ash [a]	0.9 ± 0.0%
Protein [a]	7.6 ± 0.8%
Carbohydrates [a,b]	85.1 ± 0.0%
Dietary fiber [a]	63.0 ± 2.8%
Monosaccharide composition [c]	
Rhamnose	2.8
Arabinose	12.1
Galactose	10.5
Glucose	14.6
Xylose + Mannose [d]	20.4
Uronic acids [e]	39.5

Values expressed as average ± SD.

[a] Expressed as percentage of dry weight.

[b] Calculated as 100 - (Total fat + Ash + Protein).

[c] Expressed as molar proportion (%).

[d] The two monosaccharides coeluted in HPAEC.

[e] Determined by spectrophotometric method.

Compared to the other berries, a larger portion of polysaccharides in cranberry pomace is identified as pectic polysaccharides. In addition, the relatively low proportion of rhamnose (2.8%, w/w) compared to arabinose (12.1%, w/w) and galactose (10.5%, w/w) suggests that the RG I region of cranberry pectic polysaccharides is branched with arabinan and galactan chains. This is consistent with the observed abundance of arabinans in a fraction of cranberry hull extract obtained after extensive pectinase treatment (Sun et al. 2015). Potato RG I was also reported to be highly branched with galactan (Khodaei & Karboune, 2013); while in contrast, the pectic polysaccharides from apple are predominantly composed of a HG region (A. S. Silva et al., 2014).

3.4.2. Microwave-assisted alkaline extraction

The extraction of pectic oligosaccharides from cranberry pomace by microwave-assisted alkaline method was investigated using two concentrations of KOH (0.25 and 0.5 M) and two microwave power settings (85 W/g and 242 W/g). The tested power settings were in the range applied for extraction of polysaccharides from various plant materials (Khodaei et al., 2016; Lefsih et al., 2017; A. de S. e Silva et al., 2018). Table 3.2 shows that at low microwave power setting, the yield of carbohydrate extracted was higher when high KOH molarity was used ($P<0.03$). Higher alkalinity may have loosened the bonds of polysaccharides in the cell wall, by releasing ester-bound pectin (Müller-Maatsch et al., 2016), and hence allowing more carbohydrates to be released from the cell wall. The results also indicate that at high KOH molarity of 0.5 M, the microwave power has no effect on the carbohydrate yield. High KOH molarity solution may have caused a swelling of the cell wall particles, improving the microwave absorption and limiting the difference in effect between lower and higher microwave power. These results are in agreement with the reported swelling of secondary cell wall in lignocellulosic materials (Isaac et al., 2018). Additionally, higher yields of oligo- and polysaccharides were achieved at high KOH molarity of 0.5 M ($P<0.002$). Similarly, it was reported that high alkalinity favours the microwave-assisted solubilization of polysaccharides from potato cell wall (Khodaei et al., 2016). The analysis of molecular weight distribution by HPSEC identified only oligosaccharides, indicating that the absence of water-soluble polysaccharides in the extracts while small amounts of insoluble polysaccharides may still be present in the recovered precipitates. The oligosaccharides obtained at 0.25 M KOH and 242 W/g showed a slightly lower molecular weight compared to those obtained at high alkaline molarity ($P<0.05$).

Table 3.2 Microwave-assisted alkaline extraction of oligosaccharides from cranberry pomace.

Microwave power (W/g)	KOH (M)	Carbohydrate yield [a] (%w/w)	OS and PS yield [b] (%w/w)	Molecular weight of OS (kDa)
84.9	0.25	19.4 ± 0.3 [c]	17.0 ± 1.6 [e]	1.39 ± 0.02 [c]
84.9	0.5	22.3 ± 0.6 [c]	20.9 ± 0.1 [c, d]	1.42 ± 0.03 [c]
242.4	0.25	20.4 ± 2.4 [c]	18.4 ± 0.4 [d, e]	1.18 ± 0.07 [c]
242.4	0.5	22.5 ± 0.2 [c]	22.1 ± 0.3 [c]	1.40 ± 0.02 [c]

All values are expressed as mean ± SD.

[a] Calculated as grams of carbohydrates in filtrate per gram in cranberry pomace.

[b] Calculated as grams of precipitated oligo- and polysaccharides per gram of carbohydrates in cranberry pomace.

[c-e] Sample means with different superscript letters in the same column are significantly different ($P \leq 0.05$).

The extracted oligosaccharides was estimated to be distributed around degrees of polymerization between 7 and 10. Extraction of oligosaccharides from burdock roots (J. Li et al., 2013) yielded degree of polymerization distributed around 6.

Figure 3.1 shows the monosaccharide profiles, expressed as molar proportions of the carbohydrate extracts and of the oligo/polysaccharide fraction. Compared to the cranberry pomace fiber fraction, the extracts obtained by microwave-assisted alkaline extraction showed a higher proportion of uronic acids and lower proportions of xylose, mannose and glucose ($P<0.03$). These differences were found, more marked, between cranberry pomace fiber and the oligo/polysaccharide fractions ($P<0.002$). Extracts were richer in arabinose when compared to fiber ($P<0.05$), and both extracts and oligo/polysaccharide fractions had barely detectable levels of rhamnose, inferior to what measured in the fiber ($P<0.0001$). These results indicate that microwave-assisted alkaline extraction results in the isolation of pectic oligosaccharides, specifically fragments of homogalacturonan and neutral branches of RG I, and that a large portion of the non-pectic sugars found in the extracts is composed of mono- and disaccharides rather than larger alcohol insoluble molecules. Similarly, arabinan and galactan-rich RG I were released upon alkaline treatment of potato cell wall (Khodaei et al., 2016; Zykwinska et al., 2006). The significant differences between different microwave-assisted treatments are that, in the oligo/polysaccharide fractions, low KOH concentration yielded reduced proportions of xylose and mannose, and increased that of uronic acids ($P<0.01$), while high microwave power was associated to slightly higher proportion of arabinose and galactose ($P<0.05$). Furthermore, in the total extracts, high microwave power led to slightly lower ratios of xylose and arabinose ($P<0.03$). Lower glucose was found in the extract obtained upon 0.5 M KOH-242 W/g treatment compared to the other three extracts ($P<0.008$); while higher arabinose ($P<0.04$) and galactose ($P<0.05$) proportions were found upon 0.25 M KOH-242 W/g treatment, and more xylose and mannose in 0.5 M KOH-85 W/g treatment ($P<0.05$). If compared to other oligo/polysaccharide-rich aqueous extracts from lingonberry (*Vaccinium vitis-idaea*) (Ross et al., 2015), the microwave-assisted alkali extracts from cranberry are enriched with uronic acids (53.6% vs. 30.8%), composed of very similar proportion of galactose, xylose and mannose, and showed less arabinose (20.0% vs. 30.5%) and glucose (6.3% vs. 16.6%) contents.

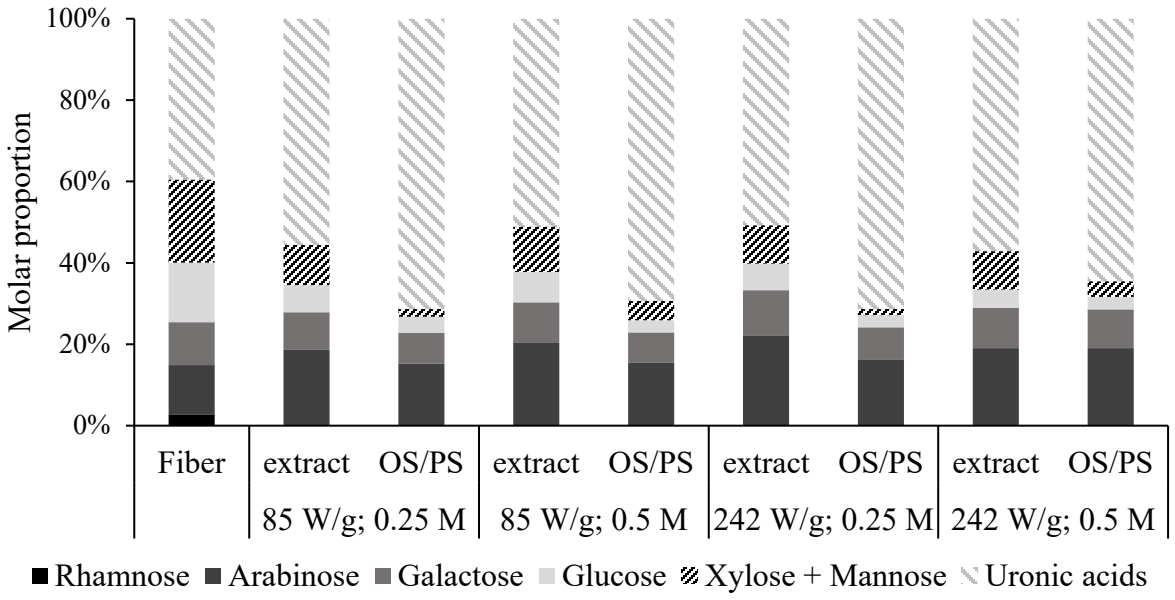

Figure 3.1 Monosaccharide profile of the microwave extracts and of the alcohol-precipitated oligosaccharides and polysaccharides (OS/PS), compared to the profile of cranberry pomace fiber.

3.4.3. Enzymatic extraction of oligosaccharides from cranberry pomace

The selective enzymatic hydrolysis of cell wall pectic polysaccharides was investigated for the extraction of oligosaccharides from cranberry pomace. However, considering the fact that the cell wall pectic polysaccharides in *Vaccinium* fruits are branched and linked to other polysaccharides (J. L. Silva et al., 2005; Stückrath et al., 1998), the efficiency of a single enzyme may be reduced by the limited substrate accessibility and by the steric hindrance. For this reason, five biocatalysts, including the pure enzyme endo-galactanase (EC 3.2.1.89) and multi-enzymatic preparations with varied levels of polysaccharide-hydrolyzing activities (including cellulase, xylanase, polygalacturonase, galactanase, arabinanase and rhamnogalacturonase), were screened for their ability to release oligosaccharides from sieved cranberry pomace. As previously reported (Khodaei & Karboune, 2016), the primary pectin-degrading activity of Depol™ 670L is galactanase, while Pectinex® Ultra SPL and Viscozyme® L have high polygalacturonase and galactanase activities; all preparations also contain arabinanase and galactanase activities. Depol™ 740L is commercialized as glucanase and contains high xylanase activity (Rommi et al., 2014), but was here determined to also possess pectin-degrading activities, including arabinanase (Table 3.3).

Table 3.4 shows the carbohydrate yields and monosaccharide profile of the extracts. All multi-enzymatic biocatalysts except for Viscozyme® L showed higher carbohydrate yields than the average of microwave-assisted alkaline extractions (21.3%) ($P<0.04$). As expected, the use of pure enzyme, endo-galactanase, gave a lower yield (not different from the yields obtained by microwaves), confirming the limitations of using a single hydrolytic activity. In comparison with the average of microwave-assisted alkali extracts, all enzymatic extracts showed lower content of galactose ($P<0.002$), and much higher content of glucose ($P<0.001$). Additionally, extracts from Pectinex® Ultra SPL, Viscozyme® L and endo-galactanase had less uronic acids, while xylose, mannose ($P<0.03$) and arabinose ($P<0.02$) were less abundant in the extracts of endo-galactanase and the Depol™ 670L.

These results indicate that a significant part of the galactose observed in the microwave-assisted alkali extraction products exists in a form scarcely accessible to enzymes and may be associated with uronic acids. It can be hypothesized that the released galactose from cranberry pomace is originated from galactan-branched pectic RG I.

Table 3.3 Enzymatic activities of multi-enzymatic preparations: main activity as reported by the producers and pectin-degrading activities measured by DNS assay after incubation at pH 5.5, 40 °C for 20 min (measurements were run in triplicates and the relative standard deviations were less than 10%).

		Enzymatic activity (U/ml) [a]			
Biocatalyst	Producers' specifications	PGase [b]	RGase [c]	Arabinanase [d]	Galactanase [e]
Pectinex® Ultra SPL	*Endo*-galacturonase	1600.5	89.9	63.2	1210.2
Viscozyme® L	Beta-glucanase	2996.4	218.1	199.6	2636.4
Depol™ 740L	Beta-glucanase	106.9	73.6	114.5	202.8
Depol™ 670L	Cellulase, *endo*-galacturonase	0.0	55.8	56.6	6751.2

[a] Unit of enzymatic activity (U) defined as the amount of enzyme that produces 1 μmol of reducing ends per min of reaction.

[b] Polygalacturonase was assessed with polygalacturonic acid from citrus fruit as substrate.

[c] Rhamnogalacturonase was assessed with rhamnogalacturonan from soybean as substrate.

[d] Arabinanase was assessed with pectic arabinan from sugar beet as substrate.

[e] Galactanase was assessed with pectic galactan from potato as substrate.

Table 3.4 Characterization of the carbohydrate extracts recovered upon enzymatic treatments of cranberry pomace.

Biocatalyst	Carbohydrate yield [a] (%w/w)	Monosaccharide profile (%mol/mol)					
		Uronic acid	Rhamnose	Arabinose	Galactose	Glucose	Xylose + Mannose
Pectinex® Ultra SPL [b]	37.6 [d, e]	23.4 [e]	0.1 [f]	16.5 [d, e]	3.4 [d]	44.4 [e]	12.3 [d]
Viscozyme® L [c]	23.4 [f, g]	18.7 [e]	1.3 [d]	19.4 [d, e]	3.6 [d]	46.7 [e]	10.3 [d]
Depol™ 740L [b]	42.0 [d]	43.3 [d]	1.0 [d]	16.3 [d, e]	2.6 [d]	30.7 [f]	6.1 [e, f]
Depol™ 670L [c]	29.8 [e, f]	44.3 [d]	0.6 [e, f]	5.1 [f]	2.2 [d]	43.7 [e]	4.1 [f]
Endo-galactanase [c]	17.7 [g]	19.8 [e]	0.0 [f]	12.9 [e]	3.9 [d]	57.9 [d]	5.5 [f]

[a] Calculated as grams of carbohydrates in extract, determined by high performance size exclusion chromatography, per gram of cranberry pomace.

[b] Reaction time 48 h.

[c] Reaction time 24 h.

[d-g] Sample means with different superscript letters in the same column are significantly different ($P \leq 0.05$).

The presence of strong interactions binding HG and RG I branches (arabinan and galactan) to the cellulose matrix in cell wall has been recently demonstrated *in vitro* and in the cell wall (Wang et al., 2018). Microwave-assisted alkaline approach may have extracted the cell wall matrix-embedded pectic galactan branches through heating and alkaline hydrolysis. The high amounts of glucose in the extract obtained upon *endo*-galactanase treatment (specific for β-1,4 linked galactosyl linkages) of cranberry pomace may have derived from water-soluble polymers initially associated with the pectic polysaccharides. Indeed, it has been reported that RG I and xyloglucan can be covalently linked in plant cell wall (Brett et al., 2005; Popper & Fry, 2008).

The extracts obtained with Viscozyme® L and Pectinex® Ultra SPL appeared similar in composition. This similarity reflects the similar levels of pectin-degrading activities measured in the two multi-enzymatic preparations. The two Depol™ formulations produced extracts with a significantly higher percentage of uronic acids compared to the other biocatalysts ($P<0.02$), despite having been reported to possess lower polygalacturonase activities compared to Viscozyme® L and Pectinex® (Khodaei & Karboune, 2016). This may be explained by the other hydrolytic activities in these mixtures that may have better exposed galacturonic acid-based polysaccharides by degrading the surrounding cell wall matrix. The proportion of neutral sugars were very similar across all extracts generated by multi-enzymatic preparations, except for Depol™ 670L, that yielded a much lower percentage of arabinose ($P<0.01$) and higher glucose ($P<0.001$); the latter can derive from the hydrolysis of cellulose and/or xyloglucan by the primary activity of the mixture (cellulase, glucanase), while the reason for reduced amount of arabinose is not apparent and is likely due to the interaction of other, non measured enzymatic activities in the mixture.

The time courses for the extractions by three biocatalysts (Depol™ 740L, Pectinex® Ultra SPL and Viscozyme® L) that were found to release the highest proportion of arabinose and galactose derived from RG I branches in pectic polysaccharides were further investigated. The biocatalysts were active at the investigated conditions (Figure 3.2), as most of the extracted carbohydrates were obtained at the initial stage of the reaction (4 h). The total yield slightly increased thereafter with Viscozyme® L, gradually decreased with Depol™ 740L, and decreased after reaching a maximum at 24 h with Pectinex® Ultra SPL.

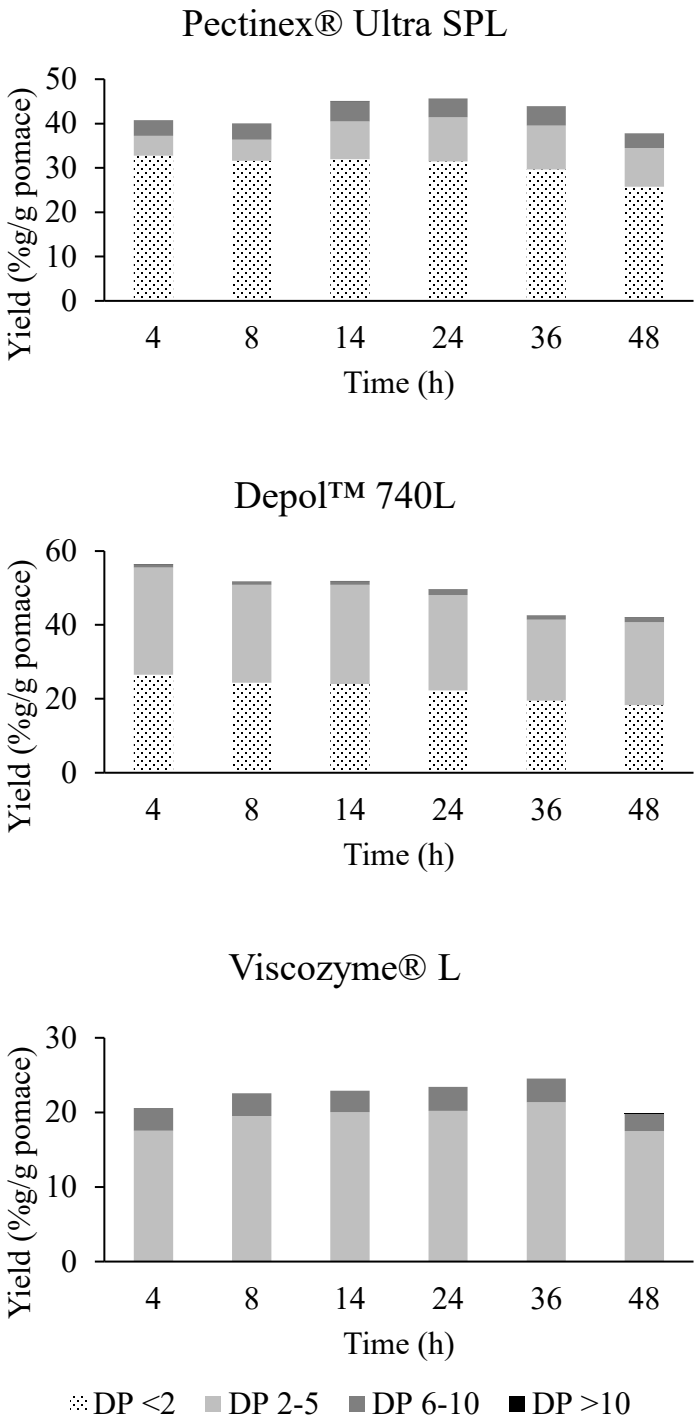

Figure 3.2 Yield of carbohydrates in the extracts obtained with selected biocatalysts, divided by degree of polymerization (DP) as determined by HPSEC.

Depol™ 740L gave the highest yield (56.5%, at 4 h), followed by Pectinex® Ultra SPL (45.5%, at 24 h). Compared to these, the maximum yield obtained from Viscozyme® L (24.6%, at 36 h) was much lower.

While all biocatalysts mainly extracted small molecular weight compounds, (DP of 2 and lower), each extract showed a distinct profile of molecular weight distribution, which changed over time. The extract obtained with Pectinex® Ultra SPL had the highest content of monosaccharides and the smallest content of short oligosaccharide (DP 2-5), whose yield slowly increased over the course of the 48 h-reaction. The content of extracted oligosaccharide with a DP between 6 and 10 instead didn't change much across the extraction process, and their proportion remained around 9.1% w/w of the extracted sugars.

The extract obtained with Depol™ 740L had the highest proportion of MS and short oligosaccharides (98.4% of the extracted sugars' weight at 4 h). The remaining 2.0% was constituted of oligosaccharides with DP in the range 6-10 and traces of larger polymers. The yield of monosaccharides and oligosaccharides with DP 2-5 showed a constant decrease over extraction time. It is possible that, with the progression of the reaction, increasing amounts of otherwise soluble products get trapped by physical retention on insoluble fragments of the cell wall matrix, released by the action of enzymatic activities and shaking.

Viscozyme® L generated an extract characterized by the absence of monosaccharides and a high percentage of short oligosaccharides that represent at all extraction times about 87% of the extracted carbohydrates. Aside from these, the extract contains mostly oligosaccharides in the DP range 6-10, and traces of polysaccharides. The proportions in the extract didn't change significantly over the course of extraction.

Figure 3.3 shows the monosaccharide profile of the extracts over time, compared with the profile of cranberry pomace fiber. In the extracts obtained upon Viscozyme® L and Pectinex® Ultra SPL treatments, uronic acid relative proportion gradually decreased from 25.1 and 32.1% at 4h to 16.1 and 23.4% at 48h, respectively; while xylose/mannose and glucose relative proportion increased by 5 to 12% over the reaction time course.

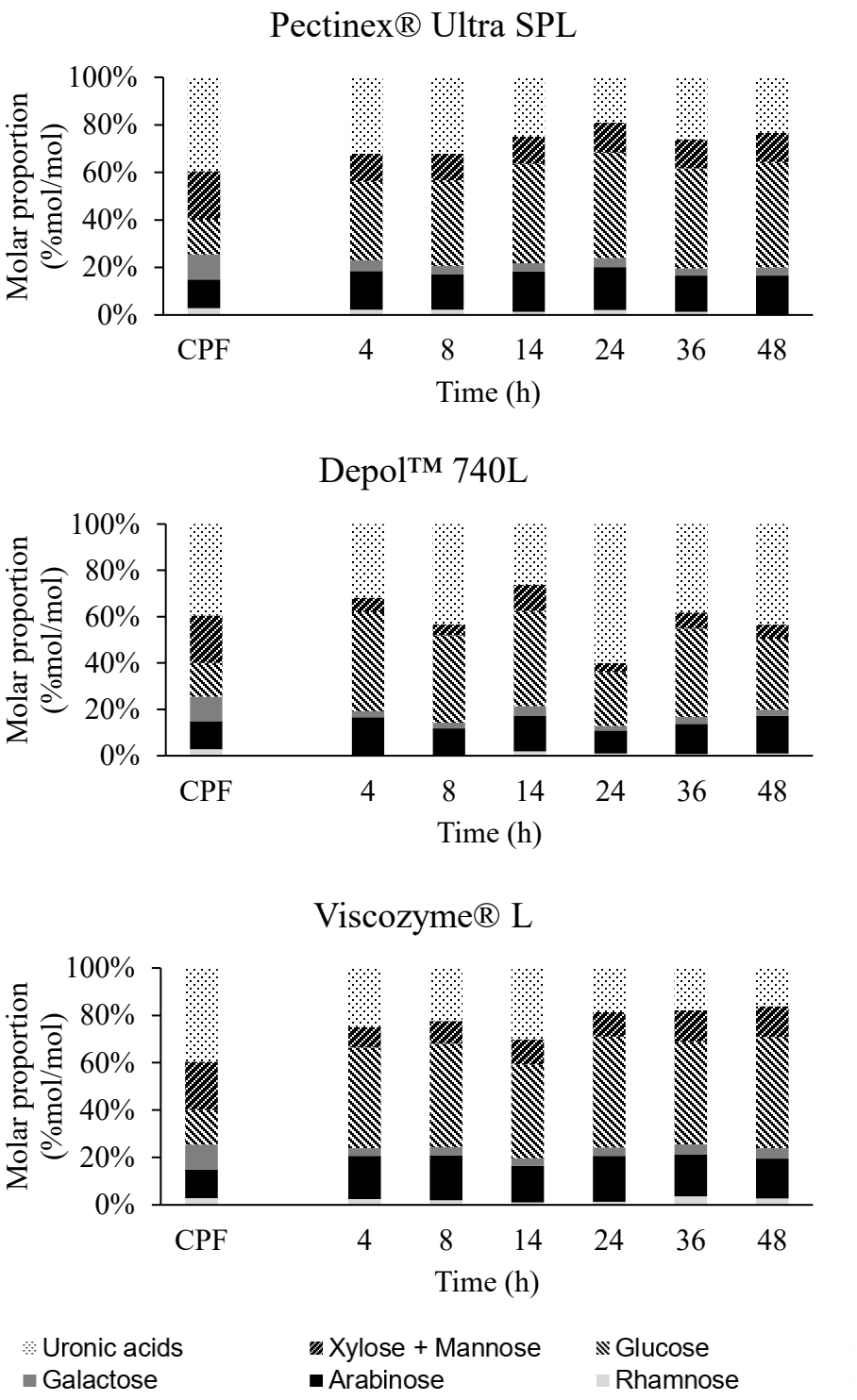

Figure 3.3 Monosaccharide profile in the supernatants obtained from enzymatic extractions, compared to the composition of cranberry pomace fiber (CPF).

These results indicate that Viscozyme® L and Pectinex® Ultra SPL liberated first from cranberry pomace mono/oligosaccharides from the cell wall pectic polysaccharides, but as the reaction time was proceeded, those derived from cellulose and hemicelluloses were released. This can be attributed to the increased activities of Viscozyme® L and Pectinex® Ultra SPL on cellulose and hemicelluloses. In contrast, the monosaccharide profile of the Depol™ 740L-based extract showed a decrease in the glucose relative proportion from 43.1 to 33.2% over the reaction time course and an increase in the uronic acid relative proportion from 32 to 43%. These results may be attributed to the low level of polygalacturonase and rhamnogalacturonase activities expressed in Depol™ 740L. Additional reaction time didn't result in increased yields, but the decrease of monosaccharides indicates that some reaction or physical change still occurred in the mixture.

3.4.4. Effect of grinding of pomace on enzymatic extraction

The morphology of pomace particles may affect the extraction of compounds (Huang et al., 2019; Bing Yang et al., 2019). To verify whether this is the case with cranberry pomace, a steel ball mill was used to prepare milled pomace. Scanning electron microscopy was then used to analyze the morphology of blended pomace and of the powders obtained from its milling for different durations. The obtained images (Figure 3.4) were used to measure the size of the cranberry pomace fragments and to qualitatively assess size distribution and porosity. Blended cranberry pomace (Figure 3.4A) appeared to be composed of fragments of cranberry skin and dry pulp with round and rectangular shapes, and dimensions around 1.6 mm. Higher magnification of the surface of one of the fragments (Figure 3.4B) showed a uniform surface with low porosity. The dimensions of the larger fragments obtained with one minute of grinding (Figure 3.4C) were found to be not much smaller than those of blended pomace (about 1.4 mm), however much smaller (0.1-0.2 mm) fragments were also observed, and the surface of the larger ones appeared more exfoliated and irregular (Figure 3.4D), similarly to the effect obtained on *Hovenia dulcis* pomace by considerably longer (2 and 6 h) ball milling (Bing Yang et al., 2019). After 5 minutes of grinding, the pomace assumed the macroscopic appearance of a fine dust, and the fragments appeared much smaller and homogenous in size (Figure 3.4E), with dimensions ranging from 0.1 mm to 0.6 mm. The surface of the bigger fragments (Figure 3.4F) looked like that of the ones obtained with one minute of grinding.

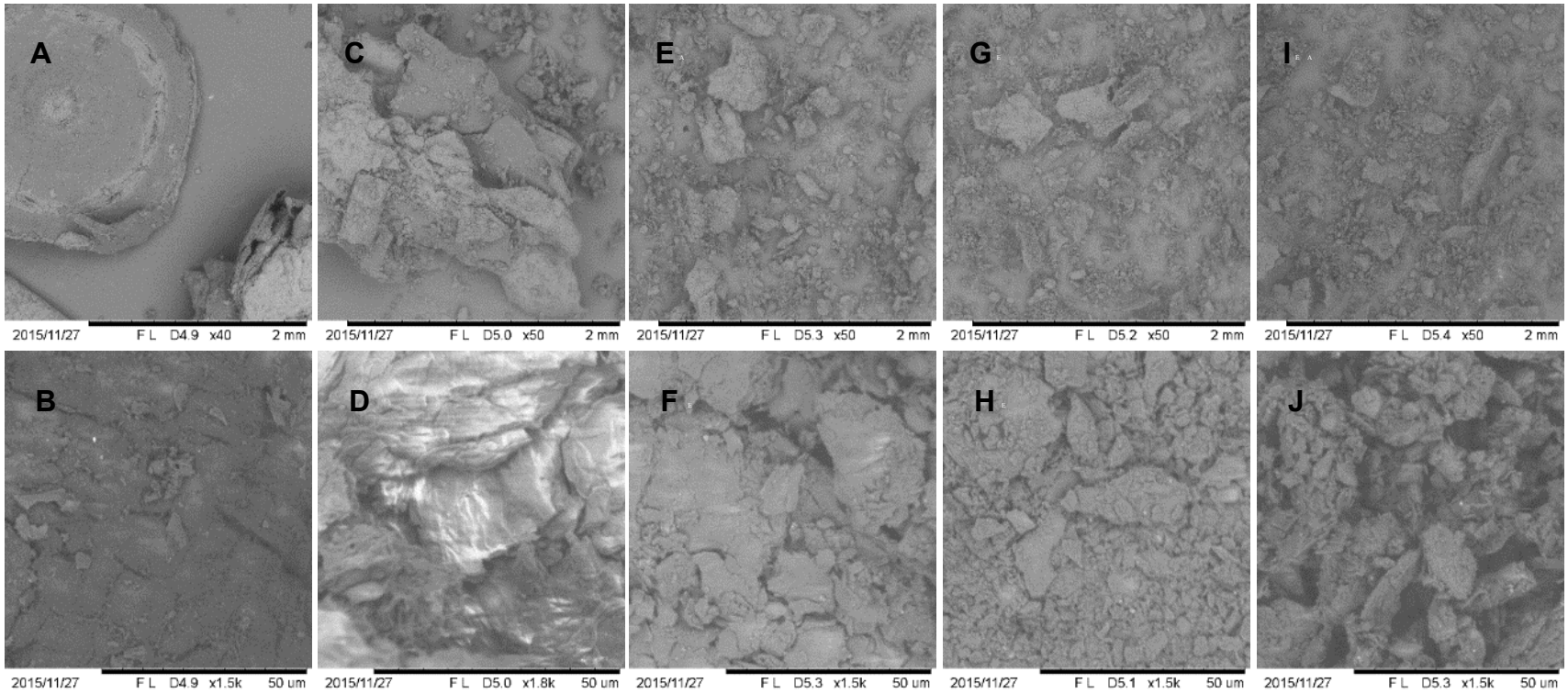

Figure 3.4 SEM images of blended and milled cranberry pomace. A, B: blended. C, D: 1 min milling. E, F: 5 min milling. G, H: 15 min milling. I, J: 30 min milling.

No appreciable reduction in fragment size or change in size distribution could be observed in the pomace ground for 15 min and 30 min (Figure 3.4G, I) when compared to 5 min treatment, while the surface of the fragments appeared more irregular (Figure 3.4H, J) with deep cracks, particularly evident in the sample obtained after 30 min of grinding.

Enzymatic extractions with Depol™ 740L were repeated in triplicate on sieved pomace, blended pomace and pomace milled for 15 min. Yield and composition of the extracts were compared, but no statistically significant difference was observed (data not shown). Hence, it was concluded that milling of the cranberry pomace doesn't affect the extraction outcome and is not advantageous under the studied extraction conditions

3.5. Conclusions

The tested microwave-assisted extraction conditions gave supernatants with similar monosaccharide profiles and carbohydrate yields (average 21.3%). At low microwave power, alkali concentration was positively correlated to the yield. Oligosaccharides with DP between 7 and 10 were obtained upon microwave-assisted extraction. In comparison with cranberry pomace fiber, the extracts resulted enriched in pectic oligosaccharides. While all enzyme-based biocatalysts produced extracts significantly enriched in glucose, those from three biocatalysts (Pectinex® Ultra SPL, Viscozyme® L and Depol™ 740L) had percentages of RG I-associated neutral sugars similar to microwave-assisted alkali extracts.

Of the three, Viscozyme® L led to the lowest yield and extracted mostly oligosaccharides with DP from 2 to 5, but displayed also the highest proportion of oligosaccharides with a DP between 6 and 10. Depol™ 740L had the highest total yield, and generated a mixture rich in oligosaccharides with DP up to 5, likely from the degradation of pectic polysaccharides and cellulose. The extract from Pectinex® Ultra SPL had an intermediate carbohydrate yield, and the highest content of sugars with DP <2. Enzymatic extractions proved viable for the hydrolysis of cranberry pomace into smaller oligosaccharides.

3.6. References

Al-Tamimi, M. A. H. M., Palframan, R. J., Cooper, J. M., Gibson, G. R., & Rastall, R. A. (2006). "In vitro fermentation of sugar beet arabinan and arabino-oligosaccharides by the human gut microflora.". Journal of Applied Microbiology *100*(2): 407–14.

Aura, A.-M., Holopainen-Mantila, U., Sibakov, J., Kössö, T., Mokkila, M., & Kaisa, P. (2015). "Bilberry and bilberry press cake as sources of dietary fibre". Food & Nutrition Research *59*(1): 28367.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). "New method for quantitative determination of uronic acids". Analytical Biochemistry *54*(2): 484–489.

Brett, C., Baydoun, E.-H., & Abdel-Massih, R. (2005). "Pectin–xyloglucan linkages in type I primary cell walls of plants". Plant Biosystems - An International Journal Dealing with All Aspects of Plant Biology *139*(1): 54–59.

Brownlee, I. A. (2011). "The physiological roles of dietary fibre". Food Hydrocolloids *25*(2): 238–250.

Caillet, S., Côté, J., Doyon, G., Sylvain, J.-F., & Lacroix, M. (2011). "Effect of juice processing on the cancer chemopreventive effect of cranberry". Food Research International *44*(4): 902–910.

Caillet, S., Lorenzo, G., Côté, J., Doyon, G., Sylvain, J.-F., & Lacroix, M. (2012). "Cancer chemopreventive effect of fractions from cranberry products". Food Research International *45*(1): 320–330.

Cashman, K. D. (2006). "A Prebiotic Substance Persistently Enhances Intestinal Calcium Absorption and Increases Bone Mineralization in Young Adolescents". Nutrition Reviews *64*(4): 189–196.

Cui, J., Lian, Y., Zhao, C., Du, H., Han, Y., Gao, W., … Zheng, J. (2019). "Dietary Fibers from Fruits and Vegetables and Their Health Benefits via Modulation of Gut Microbiota". Comprehensive Reviews in Food Science and Food Safety *18*(5): 1514–1532.

Deng, J., Shi, Z.-J., Li, X.-Z., & Liu, H.-M. (2013). "Soluble polysaccharides isolation and characterization from rabbiteye blueberry (Vaccinium ashei) fruits". BioResources *8*(1): 405–419.

Di, R., Vakkalanka, M. S., Onumpai, C., Chau, H. K., White, A., Rastall, R. A., … Hotchkiss, A. T. (2017). "Pectic oligosaccharide structure-function relationships: Prebiotics, inhibitors of Escherichia coli O157:H7 adhesion and reduction of Shiga toxin cytotoxicity in HT29 cells". Food Chemistry *227*: 245–254.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). "Colorimetric Method for Determination of Sugars and Related Substances". Analytical Chemistry *28*(3): 350–356.

Fan, H., Mazza, G., & Liao, X. (2010). "Purification, composition and antioxidant activity of polysaccharides from wolfberry, cherry, kiwi and cranberry fruits". Croatian Journal of Food Science Technology *2*(1): 9–17.

Food and Agriculture Organization of the United Nations. (2015). "FAOSTAT database".

Ganan, M., Collins, M., Rastall, R., Hotchkiss, A. T., Chau, H. K., Carrascosa, A. V., & Martinez-Rodriguez, A. J. (2010). "Inhibition by pectic oligosaccharides of the invasion of undifferentiated and differentiated Caco-2 cells by Campylobacter jejuni". International Journal of Food Microbiology *137*(2–3): 181–185.

Gouw, V. P., Jung, J., & Zhao, Y. (2017). "Functional properties, bioactive compounds, and in vitro gastrointestinal digestion study of dried fruit pomace powders as functional food ingredients". LWT *80*: 136–144.

Hancock, R. D., McDougall, G. J., & Stewart, D. (2007). "Berry fruit as "superfood": hope or hype?". Biologist *54*(2): 73–79.

Heredia, A., Jiménez, A., & Guillén, R. (1995). "Composition of plant cell walls". Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung *200*(1): 24–31.

Hilz, H., Bakx, E. J., Schols, H. A., & Voragen, A. G. J. (2005). "Cell wall polysaccharides in black currants and bilberries—characterisation in berries, juice, and press cake". Carbohydrate Polymers *59*(4): 477–488.

Huang, Y.-L., Ma, Y.-S., Tsai, Y.-H., & Chang, S. K. C. (2019). "In vitro hypoglycemic, cholesterol-lowering and fermentation capacities of fiber-rich orange pomace as affected by extrusion". International Journal of Biological Macromolecules *124*: 796–801.

Isaac, A., de Paula, J., Viana, C. M., Henriques, A. B., Malachias, A., & Montoro, L. A. (2018). "From nano- to micrometer scale: the role of microwave-assisted acid and alkali pretreatments in the sugarcane biomass structure". Biotechnology for Biofuels *11*(1): 73.

Jara-Palacios, M. J., Santisteban, A., Gordillo, B., Hernanz, D., Heredia, F. J., & Escudero-Gilete, M. L. (2019). "Comparative study of red berry pomaces (blueberry, red raspberry, red currant and blackberry) as source of antioxidants and pigments". European Food Research and Technology *245*(1): 1–9.

Jiang, Y., & Du, J. (2017). "Properties of high-methoxyl pectin extracted from "Fuji" apple pomace in China". Journal of Food Process Engineering *40*(3): e12497.

Khodaei, N., & Karboune, S. (2013). "Extraction and structural characterisation of rhamnogalacturonan I-type pectic polysaccharides from potato cell wall". Food Chemistry *139*(1–4): 617–623.

Khodaei, N., & Karboune, S. (2016). "Enzymatic generation of galactose-rich oligosaccharides/oligomers from potato rhamnogalacturonan I pectic polysaccharides". Food Chemistry *197*: 406–414.

Khodaei, N., Karboune, S., & Orsat, V. (2016). "Microwave-assisted alkaline extraction of galactan-rich rhamnogalacturonan I from potato cell wall by-product". Food Chemistry *190*: 495–505.

Lacombe, A., Wu, V. C. H., Tyler, S., & Edwards, K. (2010). "Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against Escherichia coli O157:H7". International Journal of Food Microbiology *139*(1–2): 102–107.

Latimer, G. W. J. (Ed.). (2016). *Official Methods of Analysis of AOAC INTERNATIONAL*. *Official Methods of Analysis of AOAC INTERNATIONAL* (20th ed.). AOAC International (Rockville, MD, USA).

Lefsih, K., Giacomazza, D., Dahmoune, F., Mangione, M. R., Bulone, D., San Biagio, P. L., … Madani, K. (2017). "Pectin from Opuntia ficus indica: Optimization of microwave-assisted extraction and preliminary characterization". Food Chemistry *221*: 91–99.

Li, E., Yang, S., Zou, Y., Cheng, W., Li, B., Hu, T., … Pang, D. (2019). "Purification, Characterization, Prebiotic Preparations and Antioxidant Activity of Oligosaccharides from Mulberries". Molecules *24*(12): 2329.

Li, J., Liu, X., Zhou, B., Zhao, J., & Li, S. (2013). "Determination of Fructooligosaccharides in Burdock Using HPLC and Microwave-Assisted Extraction". Journal of Agricultural and Food Chemistry *61*(24): 5888–5892.

Mtetwa, M. D., Qian, L., Zhu, H., Cui, F., Zan, X., Sun, W., … Yang, Y. (2020). "Ultrasound-assisted extraction and antioxidant activity of polysaccharides from Acanthus ilicifolius". Journal of Food Measurement and Characterization *14*(3): 1223–1235.

Müller-Maatsch, J., Bencivenni, M., Caligiani, A., Tedeschi, T., Bruggeman, G., Bosch, M., … Sforza, S. (2016). "Pectin content and composition from different food waste streams". Food Chemistry *201*: 37–45.

Olano-Martin, E., Gibson, G. R., & Rastall, R. A. (2002). "Comparison of the in vitro bifidogenic properties of pectins and pectic-oligosaccharides". Journal of Applied Microbiology *93*(3): 505–511.

Olano-Martin, Estibaliz, Rimbach, G. H., Gibson, G. R., & Rastall, R. A. (2003). "Pectin and pectic-oligosaccharides induce apoptosis in in vitro human colonic adenocarcinoma cells.". Anticancer Research *23*(1A): 341–6.

Park, S.-I., & Zhao, Y. (2006). "Development and Characterization of Edible Films from Cranberry Pomace Extracts". Journal of Food Science *71*(2): E95–E101.

Parry, J., Su, L., Moore, J., Cheng, Z., Luther, M., Rao, J. N., … Yu, L. L. (2006). "Chemical Compositions, Antioxidant Capacities, and Antiproliferative Activities of Selected Fruit Seed Flours". Journal of Agricultural and Food Chemistry *54*(11): 3773–3778.

Petkova, N., Ivanov, I., Vrancheva, R., Denev, P., & Pavlov, A. (2017). "Ultrasound and Microwave-Assisted Extraction of Elecampane (Inula helenium) Roots". Natural Product Communications *12*(2): 1934578X1701200.

Popper, Z. A., & Fry, S. C. (2008). "Xyloglucan-pectin linkages are formed intra-protoplasmically, contribute to wall-assembly, and remain stable in the cell wall". Planta *227*(4): 781–794.

Rolin, C. (1993). "Pectins". In R. Whistler & J. N. BeMiller (Eds.), *Industrial gums: Polysaccharides and Their Derivatives* (3rd ed., pp. 257–293). Academic Press (San Diego).

Rommi, K., Hakala, T. K., Holopainen, U., Nordlund, E., Poutanen, K., & Lantto, R. (2014). "Effect of enzyme-aided cell wall disintegration on protein extractability from intact and dehulled rapeseed (Brassica rapa L. and Brassica napus L.) press cakes.". Journal of Agricultural and Food Chemistry *62*(32): 7989–7997.

Ross, K. A., Godfrey, D., & Fukumoto, L. (2015). "The chemical composition, antioxidant activity and α-glucosidase inhibitory activity of water-extractable polysaccharide conjugates from northern Manitoba lingonberry". Cogent Food & Agriculture *1*(1): 1109781.

Saehu, S., Srisimarat, W., Prousoontorn, M. H., & Pongsawasdi, P. (2013). "Transglucosylation reaction of amylomaltase for the synthesis of anticariogenic oligosaccharides". Journal of Molecular Catalysis B: Enzymatic *88*: 77–83.

Saint-Denis, T., & Goupy, J. (2004). "Optimization of a nitrogen analyser based on the Dumas method". Analytica Chimica Acta *515*(1): 191–198.

Scheller, H. V., Jensen, J. K., Sørensen, S. O., Harholt, J., & Geshi, N. (2006). "Biosynthesis of pectin". Physiologia Plantarum *129*(2): 283–295.

Silk, D. B. A., Davis, A., Vulevic, J., Tzortzis, G., & Gibson, G. R. (2009). "Clinical trial: the effects of a trans-galactooligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome". Alimentary Pharmacology & Therapeutics *29*(5): 508–518.

Silva, A. de S. e, de Magalhães, W. T., Moreira, L. M., Rocha, M. V. P., & Bastos, A. K. P. (2018). "Microwave-assisted extraction of polysaccharides from Arthrospira (Spirulina) platensis using the concept of green chemistry". Algal Research *35*: 178–184.

Silva, A. S., Nunes, C., Coimbra, M. A., & Guido, L. F. (2014). "Composition of pectic polysaccharides in a Portuguese apple (Malus domestica Borkh. cv Bravo de Esmolfe)". Scientia Agricola *71*(4): 331–336.

Silva, J. L., Marroquin, E., Matta, F. B., Garner, J. O., & Stojanovic, J. (2005). "Physicochemical, carbohydrate and sensory characteristics of highbush and rabbiteye blueberry cultivars". Journal of the Science of Food and Agriculture *85*(11): 1815–1821.

Smiricky-Tjardes, M. R., Grieshop, C. M., Flickinger, E. A., Bauer, L. L., & Fahey, G. C. J. (2003). "Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations , and ileal fermentative characteristics of growing pigs". Journal of Animal Science *81*(10): 2535–2545.

Srivastava, P., & Malviya, R. (2011). "Sources of pectin, extraction and its applications in pharmaceutical industry - An overview". Indian Journal of Natural Products and Resources *2*(1): 10–18.

Stückrath, R., Pedrero, A., Tarabla, P., & Trujillo, L. (1998). "Cuantificación y caracterización de las pectinas de cranberry (Vaccinium macrocarpon)". Alimentaria (293): 23–26.

Sun, J., Marais, J. P. J., Khoo, C., LaPlante, K., Vejborg, R. M., Givskov, M., … Rowley, D. C. (2015). "Cranberry (Vaccinium macrocarpon) oligosaccharides decrease biofilm formation by uropathogenic Escherichia coli". Journal of Functional Foods *17*: 235–242.

USDA - National Agricultural Statistics Service. (2017). *Noncitrus Fruits and Nuts 2016 Summary*.

Vigsnæs, L. K., Holck, J., Meyer, A. S., & Licht, T. R. (2011). "In Vitro Fermentation of Sugar Beet Arabino-Oligosaccharides by Fecal Microbiota Obtained from Patients with Ulcerative Colitis To Selectively Stimulate the Growth of Bifidobacterium spp. and Lactobacillus spp.". Applied and Environmental Microbiology *77*(23): 8336–8344.

Voragen, A. G. J., Coenen, G.-J., Verhoef, R. P., & Schols, H. A. (2009). "Pectin, a versatile polysaccharide present in plant cell walls". Structural Chemistry *20*(2): 263–275.

Wang, D., Yeats, T. H., Uluisik, S., Rose, J. K. C., & Seymour, G. B. (2018). "Fruit Softening: Revisiting the Role of Pectin". Trends in Plant Science *23*(4): 302–310.

White, B. L., Howard, L. R., & Prior, R. L. (2010). "Proximate and Polyphenolic Characterization of Cranberry Pomace". Journal of Agricultural and Food Chemistry *58*(7): 4030–4036.

Yang, B., Wu, Q., Song, X., Yang, Q., & Kan, J. (2019). "Physicochemical properties and bioactive function of Japanese grape (Hovenia dulcis) pomace insoluble dietary fibre modified by ball milling and complex enzyme treatment". International Journal of Food Science & Technology *54*(7): 2363–2373.

Zykwinska, A. W., Rondeau-Mouro, C., Garnier, C., Thibault, J.-F., & Ralet, M.-C. (2006). "Alkaline extractability of pectic arabinan and galactan and their mobility in sugar beet and potato cell walls". Carbohydrate Polymers *65*(4): 510–520.

CONNECTING STATEMENT 2

In chapter III the composition of cranberry pomace from industrial processing was analyzed, and different approaches for the extraction of oligosaccharides from cranberry pomace were investigated. The obtained extracts were characterized by the presence of monosaccharide residues associated to both pectic polysaccharides and hemicellulose, in variable proportions. To determine the properties and abundance of the different cranberry cell wall polysaccharides, compounds that had received little attention compared to the fruit's polyphenols, the study presented in chapter IV was conducted. Whole ripe Stevens variety cranberries, one of the main varieties used for juice production, were acquired and their cell wall solids were extracted with a sequence of aqueous solvents targeted at selectively solubilizing polysaccharides with different chemical properties. The extracts, fractioned by anion exchange chromatography, were characterized in terms of molecular weight distribution, monosaccharide profile and glycosidic linkage.

The results from this study were presented at Biotrans 2017 – 13th International Symposium on Biocatalysis & Biotransformations and submitted to a scientific journal.

Spadoni Andreani, E., Liu, L. & Karboune, S. (2020). Extraction and Characterization of Cell Wall Polysaccharides from Cranberry (*Vaccinium macrocarpon* var. Stevens) Pomace. Carbohydrate Polymers (Submitted).

CHAPTER IV. EXTRACTION AND CHARACTERIZATION OF CELL WALL POLYSACCHARIDES FROM CRANBERRY (*VACCINIUM MACROCARPON* VAR. STEVENS) POMACE

4.1. Abstract

Cranberries of Stevens variety, mainly used for juice production, were processed into pomace, from which alcohol insoluble solids were obtained. A sequence of four extractions methods was applied to the solids. The extracts were compared in terms of monosaccharide profile, molecular weight distribution and sugar linkage. The extracts obtained by hot buffer, chelating agents and diluted alkali were found to be enriched in pectic polysaccharides, while the concentrated alkali one mostly contained hemicelluloses. All extracts contained high molecular weight polysaccharides (>10^5 Da). Chelating agents extract had the highest yield (11.0%, w/w of solids) as well as the highest abundance of uronic acids (83.4% mol%). These less tightly bound cell wall pectic polysaccharides were characterized by a molar ratio of Ara to Gal of 1:1 to 2.2:1. Linkage analysis revealed the presence of abundant arabinan and type II arabinogalactan, while galactan and type I arabinogalactan were found in lower amounts.

4.2. Introduction

Cranberry pomace is a very abundant residual material from juice production. Its composition, in particular its polyphenols and cell wall polysaccharides, has attracted a high interest. Polyphenols, despite not very abundant (0.6%, w/w) (Vattem & Shetty, 2002) are rich in A-type proanthocyanidins that have shown high antioxidant properties (Abeywickrama et al., 2016) and are promising antibiofilm compounds against urinary tract infections (Singh et al., 2016). Cell wall polysaccharides represent most of cranberry pomace solids (75%, w/w) (White et al., 2010b) and can act as dietary fibers and as a source of prebiotics that can modulate the gut microbiota and promote intestinal health. They also have an array of techno-functional properties including gel formation (Kaya et al., 2014) and emulsion stabilization (Kolanowski et al., 2004) that make them valuable food ingredients, with applications such as fat replacement (Zbikowska et al., 2018).

There is an increasing demand for the effective use of the cranberry pomace as source of functional ingredients of industrial interest. Pilot-scale extraction of polyphenols from cranberry pomace has been reported (Harrison et al., 2013), but its cell wall polysaccharides have received less attention. In fact, only a limited number of studies has quantified and characterized the polysaccharides of Vaccinium genus fruits (Clague & Fellers, 1934; Deng et al., 2013; Elwell & Dehn, 1939; Fan et al., 2010; Hilz et al., 2005, 2006), and the structural features of cranberry polysaccharides have not been reported in detail.

The sequential extraction of cell wall polysaccharides using series of different aqueous extractants has proven effective for isolating and understanding the distribution of pectic and hemicellulosic polysaccharides within the cell wall materials of other small berries, apple, citrus, olive and soybean (Hilz et al., 2005; Kosmala et al., 2010; Prabasari et al., 2011; Vierhuis et al., 2000; Villanueva-Suárez et al., 2013). To the authors' knowledge, the present study is the first to describe the application of the sequential approach for extracting cranberry cell wall polysaccharides. The extracts were characterized in terms of molecular weight distribution, monosaccharide composition and glycosidic linkage. Further fractionation of the extracts was carried in order to recover and characterise the pectic polysaccharides-enriched fractions. Stevens variety, used in the present study, is one of the main commercial cultivars of cranberry used for juice production; this variety is characterised by the comparatively higher content of sugars and less tannic flavor due to reduced content of phenolics (Narwojsz et al., 2019). Our study is expected to contribute to the understanding of cranberry cell wall polysaccharides at molecular and structural levels that can provide guidance for their application as food ingredients.

4.3. Materials and methods

4.3.1. Preparation of cranberry pomace and alcohol insoluble solids

Stevens variety cranberries were provided by Les Canneberges Atoka Inc., Manseau, QC, Canada. Cranberries (1000 g) were blended in a Vitamat juicer (Rotor Lips AG, Switzerland). The solids were collected, and the juice was extracted by pressing in cheese cloth. The remaining pomace was freeze dried, blended and sieved into a fine powder (< 1.18 mm). Alcohol insoluble solids (AIS) were obtained by suspending the powder in 95% ethanol at a concentration of 13.8 % w/v. The suspension was shaken for 1 h at 150 rpm in an Excella E24 orbital shaker (New Brunswick Scientific, USA) before filtering on Miracloth rayon-polyester cloth (Millipore Sigma, USA). AIS were washed three times with 85% ethanol, chloroform:methanol (1:1, v:v) and acetone, and dried overnight at room temperature.

4.3.2. Sequential extraction of polysaccharides

To isolate polysaccharides from AIS, a four-step sequential extraction method was used (Figure 4.1), according to a modification of the procedure described by Hilz et al. (2005). Hot buffer extract (HB) was obtained by suspending dry AIS (4%, w/v) in 200 ml of buffer A (0.05 M sodium acetate buffer, pH of 5.2) at 70 °C for 10 min for three times. The recovered solids were suspended (2.66%, w/v) in 0.05 M ethylenediaminetetraacetic acid, 0.05 M sodium oxalate in buffer A at 70 °C for 15 min.

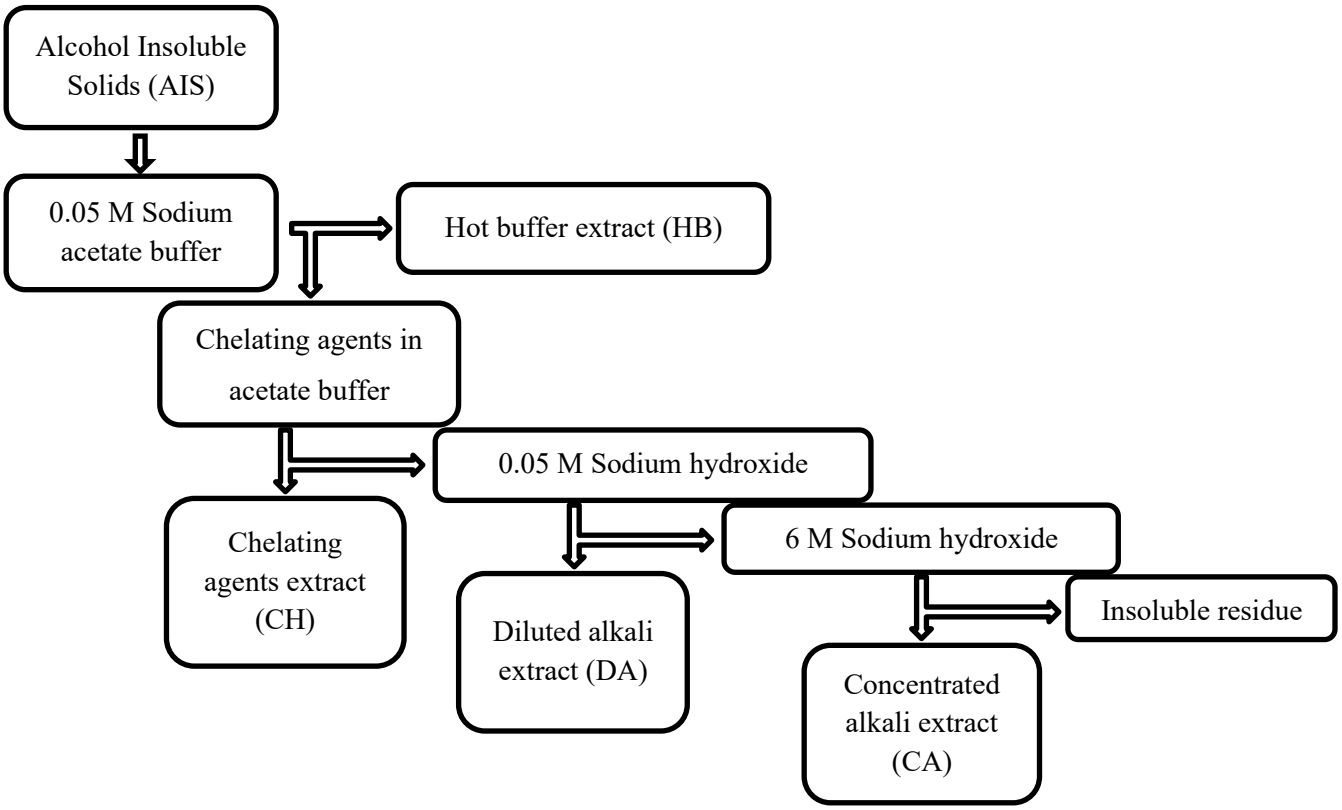

Figure 4.1 Sequential extraction diagram.

This extraction step was repeated twice, obtaining the chelating agents extract (CH). Solids were washed with water to remove excess chelating agents, then suspended in 0.05 M sodium hydroxide (2.66%, w/v) at 0 °C for 60 min to yield diluted alkali extract (DA). Finally, concentrated alkali extract (CA) was obtained by extracting twice with 6 M NaOH (2.66%, w/v) at 0 °C for 60 min. After each extraction step, the suspensions were brought to pH 5.2 with acetic acid, centrifuged at 8000 ×G for 30 min and filtered on fritted glass funnel of medium pore size. The supernatants were pooled and concentrated by ultrafiltration with a Prep/Scale spiral wound tangential flow cartridge (Millipore Sigma, USA). The retentates were dialyzed with a cut-off of 5-8 kDa against water (HB, DA, CA) or 0.1 M sodium chloride followed by water (CH). The insoluble residue recovered at the end was washed twice with water (3.2%, w/v) and freeze dried.

4.3.3. Fractionation of polysaccharides extracts by anion exchange chromatography

The extracts were fractioned by anion exchange chromatography using an ÄKTApurifier UPC 10, on Source 15Q column (GE Healthcare, Chicago, IL, USA). Elution was conducted at constant flow rate 1.5 ml/min with one column volume of 0.005 M sodium acetate buffer at pH 5, followed by a linear gradient for 7 column volumes up to 1.5 M. Fractions containing high concentrations of uronic and/or neutral sugars were pooled, dialyzed and freeze dried.

4.3.4. Protein content determination

The protein content of the extracts was determined by colorimetric test as described by (Bradford, 1976) using bovine serum albumin as standard using the assay kit from Bio-Rad (Hercules, CA, USA).

4.3.5. Sugar content and monosaccharide composition analyses

Uronic acid (UA) content was measured by sulphamate/m-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973). The phenol–sulphuric acid assay was used for the determination of neutral sugar content (DuBois et al., 1956). To determine the monosaccharide composition, samples were first hydrolyzed using a two-step procedure as previously described by Khodaei & Karboune (2013). The samples were suspended and incubated at 60 °C for 24 h in HCl/methanol mixture (1:4, v/v) at a ratio of 0.6% (w/v) and thereafter boiled for 1 h in trifluoroacetic acid solution at a ratio of 1:8 (v/v). Hydrolyzed samples were analyzed with high performance anionic exchange chromatography, equipped with pulsed amperometric detector (ICS 3000, Dionex Co., Sunnyvale, CA, USA), and a CarboPac PA20 column (3×150 mm).

Isocratic elution was performed with 5 mM NaOH (0.5 ml/min). L-Rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-xylose (Xyl) and D-mannose (Man) were used as standards.

4.3.6. Analysis of glycosidic linkages

A portion of the polysaccharides extracts was subjected to the carboxyl reduction of the galacturonic acid (GalA) residues to Gal as described by Taylor and Conrad (1972), followed by methylation to partially methylated alditol acetates (PMAAs) as described by Anumula and Taylor (1992). Samples (0.5 µl) were injected in splitless mode in a gas chromatograph (Agilent, Santa Clara, CA, USA) with Agilent DB-5HT column (30 m×250µm×0,1µm). Linkage type was determined by comparing the EI-MS fragment pattern with NIST library and the PMAAs database from the Complex Carbohydrate Research Center (https://www.ccrc.uga.edu/specdb/ms/pmaa/pframe.html). GalA linkages were determined by comparing the linkage proportions of reduced extracts with those of extracts subjected to methylation without carboxyl reduction step.

4.3.7. Molecular weight distribution analysis

A high-performance size-exclusion chromatography (HPSEC) system (Model 1525, Waters Co., Milford, MA, USA) was used to estimate the molecular weight distribution and to quantify the yield of polysaccharides extraction. Columns TSK G5000 PWXL and TSK G3000 PWXL (Tosoh Co, Yamaguchi, Japan) were used in series with isocratic flow rate of 0.4 ml/min of 0.1 M sodium chloride. Refractive index detector was operated at 30 °C. Dextrans (50-670 kDa) and soybean rhamnogalacturonan standards (0.3 - 5 g/l) were used for calibration. Yield of extraction was calculated by dividing the summed weight of the compounds eluted in HPSEC by the initial weight of AIS.

4.3.8. NMR spectroscopy

^{1}H (800 MHz) and ^{13}C NMR (200 MHz) spectra were recorded on an AVANCE III HD spectrometer (Bruker Corp., Billerica, MA, USA) with TCI cryoprobe for 3–5 % solutions of polysaccharides in D_2O (100 % D) at 328 K. Dry polysaccharide fractions were dissolved (10-15 mg) in 500 µL of D_2O and centrifuged to remove insoluble residue. Chemical shifts were referenced to internal sodium 4,4-dimethyl-4-silapentan-sulfonate (DSS) (Sigma-Aldrich Co., St. Louis, MO, USA). Two-dimensional heteronuclear spectra (HSQC and HSQC-TOCSY) were recorded using standard Bruker procedures. Three-dimensional HSQC-TCOSY data was collected using 25% non-uniform sampling.

Data were processed with NMRPipe (Delaglio et al., 1995) and visualized with NMR View (Johnson & Blevins, 1994). Chemical shifts were interpreted in carbohydrate structure context by comparison with literature data (Makarova et al., 2018; Shakhmatov et al., 2019; Zou et al., 2020) as well as with data collected and averaged in Carbohydrate Structure Database (Toukach & Egorova, 2016).

4.3.9. Statistical analysis

Statistical analyses were performed using XLSTAT software (Addinsoft, New York, NY, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test were performed to detect significant differences ($P < 0.05$).

4.4. Results and discussion

4.4.1. Characterization of alcohol insoluble solids

From 1000 g of whole Stevens cranberries 32.4 g of dry pomace were obtained on average (Table 4.1), a value consistent with the reported dietary fiber content of fresh cranberry (United States Department of Agriculture & Agricultural Research Service, 2019). The cranberry AIS represented 80.2% w/w of dry pomace. The content of AIS is consistent with the removal of protein, fat and ash, representing 14.8% (Chapter III). Furthermore, this AIS content is in coherence with the total fiber contents reported for cranberry in literature, ranging from 58.7 to 74.8% (Masli et al., 2018; White et al., 2010b), and with the ranges for apple and black currant pomaces (Kosmala et al., 2010). The relatively large ranges of this value may be attributed to the analytical techniques, as well to differences in the cultivar, ripeness and industrial processing. Whole Stevens variety cranberries contained on average 2.6% w/w of AIS, showing no statistical difference with the value (3.8%) reported for bilberry (Hilz et al., 2005). Monosaccharide composition of the AIS (Table 4.1) provided a first characterization of its cell wall polysaccharides. Uronic acids (UA) represented the major monosaccharide moiety, with a relative molar proportion of 52.4% (mol%). This suggests that cranberry cell wall material is richer in pectic polysaccharides than apples, olives and oranges, whose AIS were reported to contain only 16-28%, 23% and 44% of UA, respectively (Kosmala et al., 2010; Prabasari et al., 2011; Renard & Thibault, 1993; Vierhuis et al., 2000). It is worth to note that apple and orange cell walls are commonly used as sources for the extraction of pectin.

Table 4.1 Yield and monosaccharide profile of alcohol insoluble solids (AIS) present in pomace of whole Stevens cranberries.

Pomace (w/w of whole Stevens cranberries)	3.24 ± 0.3%
AIS (w/w of dry pomace)	80.2 ± 8.0%
AIS (w/w of whole Stevens cranberries)	2.6 ± 0.4%
Monosaccharide Composition of AIS (mol %)	
- Rhamnose (Rha)	2.8 ± 0.0
- Arabinose (Ara)	14.7 ± 1.6
- Galactose (Gal)	9.8 ± 0.9
- Glucose (Glc)	9.4 ± 1.0
- Mannose (Man)+ Xylose (Xyl)	10.9 ± 1.8
- Uronic acids (GalA) [a]	52.4 ± 1.5

All values are expressed as mean ± SD.

AIS: alcohol insoluble solids.

[a] Uronic acid content was determined by m-hydroxydiphenyl colorimetric method.

Comparing with the monosaccharide profiles of bilberry pomace and AIS (Aura et al., 2015; Hilz et al., 2005), cranberry contained significantly higher ($P<0.033$) molar percentages of Rha (1% in bilberry), Ara (5% in bilberry) and UA (15% in bilberry), which are three major constituents of pectic polysaccharides, while the sugars associated with hemicelluloses and cellulose (Man, Xyl and Glc) were less abundant in cranberry ($P<0.05$). Hence, despite cranberry and bilberry are part of the same genus *Vaccinium*, cranberry appeared to possess a higher content of cell wall pectic polysaccharides than bilberry. The monosaccharide profile of cranberry AIS was in fact closer to that of black currant (Hilz et al., 2005) and orange albedo (Prabasari et al., 2011), with whom no statistically significant difference was observed. These results indicate that cranberry pomace AIS are a pectic polysaccharides-rich material compared to other commercially relevant pomaces and to other similar berries.

4.4.2. Sequential extraction and molecular weight distribution of cell wall polysaccharides

The yields of sequential extractions (Table 4.2) ranged from 3.0% (CA) to 11.0% (CH); while the yield of insoluble residues was 67.5%. Total yield of extracted polysaccharides (21.8% of AIS dry weight) was comparable with the one obtained for bilberry (19%), and black currant AIS (30.6%) by Hilz et al. (2005), but lower ($P=0.001$) than that from apple pomace (64%) (Ma et al., 2019). Cranberry cell wall proved, therefore, richer in insoluble materials than apple. Studies on cell wall materials extracted from olive, bilberry, black currant and orange (Hilz et al., 2005; Prabasari et al., 2011; Vierhuis et al., 2000) reported that HB contains the less tightly bound cell wall polysaccharides, while CH contains pectic polysaccharides that were bound by ionic bonds, for instance via calcium ions (Ralet et al., 2003). As the yield of cranberry CH was 2.5 times higher than that reported for the CH from bilberry (Hilz et al., 2005), such ion-bound polysaccharides may be more abundant in cranberry than in bilberry.

Most extracts showed polydispersity in their molecular weight distribution (Figure 4.2). In HB two main populations at ~1.5×10^6 Da and 10^5 Da were obtained, with the largest molecular weight peak representing 56% (w/w) of total HB polysaccharides. In CH, three populations were found, distributed around molecular weights of 1.8×10^6 Da (42%, w/w), 10^5 Da (21%, w/w) and ~1.5×10^3 Da (37%, w/w), the last being indicative of the presence of oligosaccharides. The polysaccharides present in the CA were distributed in two more defined peaks, centered at 8.9×10^5 (66%, w/w) and 1.3×10^4 Da. Finally, DA was the only extract where polysaccharides represented a single peak, centered at 3.2×10^5 Da. The molecular weights of polysaccharides in bilberry and black currant AIS extracts (Hilz et al., 2005) were reported to be higher in HB and CH, similarly to what was observed in cranberry.

Table 4.2 Extraction yield and monosaccharide profile of extracts and insoluble residue upon the sequential extraction of Stevens variety alcohol insoluble solids (AIS).

	Yield (g/100 g AIS)	Protein content %	Monosaccharide profile (mol%)					
			Rha	Ara	Gal	Glc	Man + Xyl	UA
HB	3.2	13	2.3 ± 0.3^{c}	10.5 ± 0.1^{c}	5.4 ± 0.8^{d}	6.1 ± 0.5^{d}	2.8 ± 0.2^{d}	72.8 ± 0.5^{c}
CH	11.0	8	1.1 ± 0.1^{c}	7.1 ± 0.1^{c}	3.5 ± 0.7^{e}	2.8 ± 0.1^{d}	2.1 ± 0.1^{d}	83.4 ± 0.4^{b}
DA	4.5	<1	1.6 ± 0.1^{c}	13.8 ± 0.4^{bc}	6.2 ± 0.3^{d}	1.7 ± 0.9^{d}	1.6 ± 0.6^{d}	75.2 ± 0.3^{c}
CA	3.0	<1	1.8 ± 0.0^{c}	12.9 ± 1.7^{c}	13.8 ± 0.2^{b}	35.9 ± 4.7^{b}	30.2 ± 1.6^{b}	5.5 ± 0.4^{f}
Insoluble Residue	67.5	ND	5.9 ± 0.0^{b}	21.7 ± 2.3^{b}	14.6 ± 0.9^{b}	16.5 ± 3.4^{c}	13.4 ± 2.1^{c}	28.0 ± 0.9^{e}

All values are expressed as mean ± SD. ND: not determined.

HB: hot buffer extract; CH: chelating agents extract; DA: diluted alkali extract; CA: concentrated alkali extract; L-Rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-xylose (Xyl), Uronic acids (UA) and D-mannose (Man).

a determined by m-hydroxydiphenyl colorimetric method.

b-f Within the same column, means with different letters are significantly different at $P \leq 0.05$.

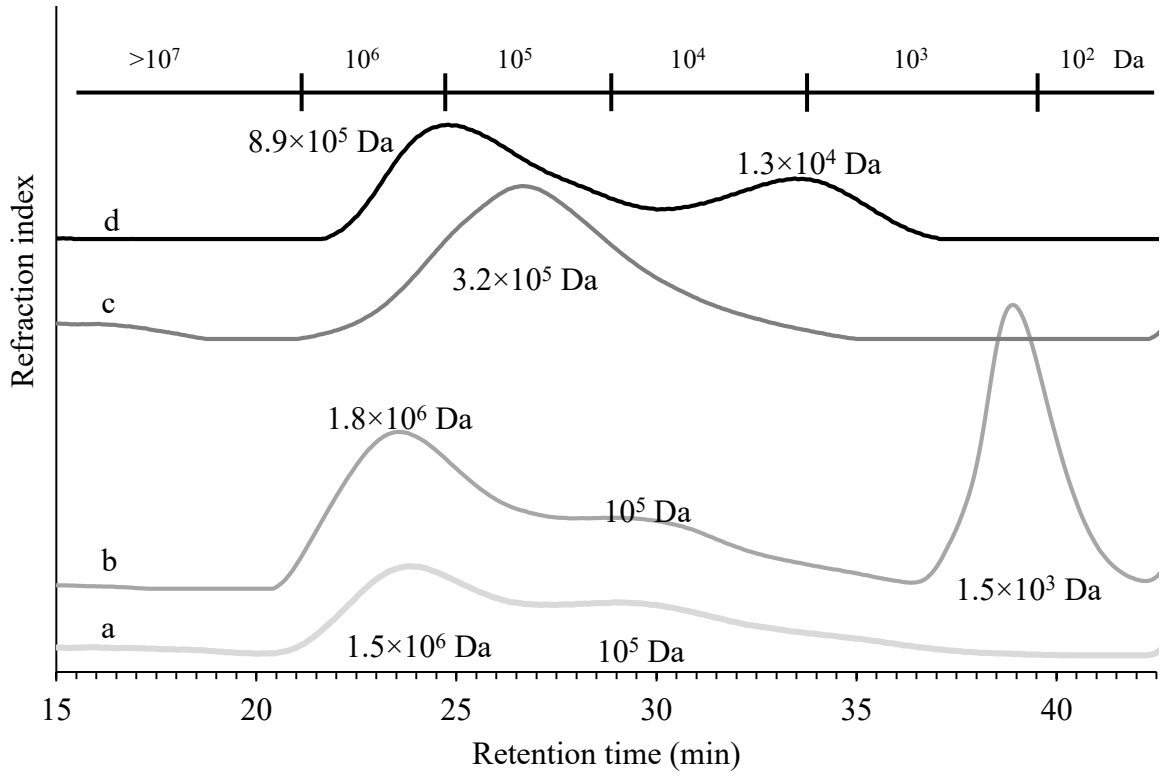

Figure 4.2 Molecular weight distribution in the extracts obtained from alcohol insoluble solids. a: Hot buffer extract; b: Chelating agents extract; c: Diluted alkali extract; d: Concentrated alkali extract.

However, contrary to cranberry cell wall polysaccharides extracts, no oligosaccharide peak was reported in the bilberry and black currant CH, and their polysaccharides in DA were distributed around higher molecular weights than the ones in CA (Hilz et al., 2005). In another fruit of the same genus, rabbiteye blueberry, polysaccharides with smaller molecular weights were isolated, the smallest being in DA and CA (4.3×10^3, 8.3×10^3 Da), and the largest in CH and HB (50.9×10^3, 122.5×10^3 Da) (Deng et al., 2013).

4.4.3. Monosaccharide composition of cranberry cell wall polysaccharides

As shown in Table 4.2, UA residues were at high abundance in the first three extracts: HB (72.8%, mol%), CH (83.4%) and DA (75.2%). Previous studies have reported high percentages of UA in the HB and CH of olive (65%, 66%), bilberry (80%, 85%), black currant (78%, 83%), orange albedo (84%, 85%), apple (60%, 67%), beet root (61%, 58%) and black tomato (71%, 85%) (Coimbra et al., 1994; Hilz et al., 2005, 2006; Kosmala et al., 2010; Prabasari et al., 2011; Renard & Thibault, 1993; Renard et al., 1990; W. Zhang et al., 2020). The monosaccharide composition of cranberry CH was very similar to the one reported for CH from non-enzymatically treated apple pomace, black currant (Hilz et al., 2005, 2006; Kosmala et al., 2010), bilberry (Hilz et al., 2005, 2006) and orange albedo (Prabasari et al., 2011). The prevalence of UA in the CH fitted with the physical basis of the chelating agent extraction that aims at isolating pectic polysaccharides by removing the bridges between metal ions and their acidic sugar residues. The high uronic acid content of cranberry DA (75.2%, mol%) reveals the presence of some pectic polysaccharides that are covalently linked to cranberry cell wall polysaccharides through covalent ester bonds (Christiaens et al., 2012). A previous study also shows that sodium carbonate solubilized pectic polysaccharides from rabbiteye blueberry (56% uronic acid content, w/w) (Deng et al., 2013). DA from other vegetal sources were reported to contain lower UA, such as orange albedo (25%) (Prabasari et al., 2011) and carrot purées (20%) (Christiaens et al., 2012), while apple (Kosmala et al., 2010) and black tomato (W. Zhang et al., 2020) had similar contents to cranberry DA.

Compared to the literature, the molar proportion of Glc residues in cranberry HB is higher than those reported for several other fruit cell wall HB, except for non-enzymatically treated apple pomace, rabbiteye blueberry and black tomato (Deng et al., 2013; Kosmala et al., 2010; W. Zhang et al., 2020). Among the four cranberry extracts, the lowest amount of Ara and Gal were found in the CH, suggesting that the abundant pectic polysaccharides that it contains possess fewer neutral regions, or neutral regions with less and/or shorter arabinan branches compared to the other extracts.

The results also show that in the CA, UA residues represented only 5.5% of the monosaccharides, a much lower content compared to values reported in CA of bilberries (16%) and olives (26%) (Hilz et al., 2005, 2006; Vierhuis et al., 2000), but higher than rabbiteye blueberry (2%), whose CA contained almost exclusively non-pectic monosaccharides, with a particularly high amount of Xyl (Deng et al., 2013).

Coherently with a low level of pectic polysaccharides, the most represented monosaccharide residues in cranberry CA were Glc (35.9%) and Man/Xyl (30.2%), that can be originated from xyloglucan, heteroxylans and heteromannans. Noticeably, Ara (12.9%) and Gal (13.8%) were also found in this CA. In the literature, higher abundance of Ara in CA was reported only for orange albedo (19%), which contained a similar amount of Gal (14%), and olive cell wall, in which Ara alone accounts for 42% of DA, while Gal is less abundant (7%) (Prabasari et al., 2011; Vierhuis et al., 2000). Considering their relatively high proportion, Ara and Gal residues in the cranberry CA may not entirely derive from decorations of hemicelluloses, but also from arabinan and galactan chains, such as the neutral branches of pectic polysaccharides. These structures, if strongly bound to the hemicelluloses, may have resisted to the extraction with diluted sodium hydroxide (Popper & Fry, 2008). This hypothesis is also supported by the higher molar ratio of Rha to UA in CA (32:100) compared to the first three extracts (2:100). The presence of Rha in cell wall is strongly connected to the rhamnogalacturonan I (RGI) backbone, that consists of repeating residues of α-1,4-D-GalA and α-1,2-L-Rha, to which side chains containing Gal and/or Ara residues are attached.

The monosaccharide compositions of cranberry, bilberry (Hilz et al., 2005, 2006) and rabbiteye blueberry (Deng et al., 2013) extracts were compared, as the three species belong to the same *Vaccinium* genus. The only statistically significant difference observed with bilberry ($P=0.01$) is that cranberry has a higher proportion of Ara in CA. On the other hand, cranberry was found to contain less Ara and Glc in both HB and CH compared to rabbiteye blueberry ($P<0.05$), as well as less total non-pectic sugars (Xyl, Man and Glc) in DA ($P<0.0001$) and more Ara in CA ($P=0.013$). However, it was observed that the extracts from cranberry exhibited a higher molar ratio of Ara to Gal (1:1 to 2.2:1) than the corresponding bilberry extracts (0.25:1 to 1.25:1). This suggests that the neutral branches of pectic polysaccharides in the two fruits may subtly differ in composition, with Ara-containing polysaccharides found in greater amounts in cranberry.

4.4.4. Anionic fractionation of cranberry cell wall polysaccharides

The cranberry cell wall polysaccharides extracts were fractionated by anionic exchange chromatography. All profiles (Figure 4.3) displayed a peak corresponding to neutral polysaccharides with absence or very low levels of UA in the fractions eluted at low buffer concentration (0.005 M). The elution profiles reveal a major uronic acid-rich peak in both HB and CH (HB-1 and CH-1, respectively) eluted at 0.414 M, associated with a small amount of neutral sugars. Additionally, CH had a second uronic acid-rich peak (CH-2) eluted at 0.958 M. The fractionation of DA revealed three peaks at relatively low buffer concentration. DA-1 peak was eluted at 0.522 M containing both neutral sugars and uronic acid residues; this DA-1 peak might represent a fraction containing pectic polysaccharides with neutral branches, such as RGI. The other two peaks (DA-2) rich in uronic acid residues were eluted at 0.846 and 1.078 M. For CA, only one peak (CA-1) composed mainly of neutral sugars was observed, at the initial ionic strength. This result confirms that in CA pectic polysaccharides are scarce and may be limited to short RGI bound to hemicelluloses. In comparison, CH and DA extracts from soybean showed nearly identical elution profiles, both appearing very similar to cranberry CH but with a more marked neutral sugars peak eluted at initial ionic strength (Huisman et al., 1998).

Selected fractions (HB-1, CH-1, CH-2, DA-1, DA-2, CA-1) were analyzed for their molecular weight distribution and monosaccharide profile. Figure 4.4 shows that HB-1 consisted of a polysaccharide peak distributed around 9.6×10^5 Da, which is likely to be a subset of the population (1.5×10^6 Da) identified in the whole HB (Figure 4.2). CH-1 and CH-2 fractions had similar compositions, with one peak at 1.3×10^6 Da (94%, w/w) and a small one at 2.5×10^3 Da. Both peaks appear to belong to the two populations identified at 1.8×10^6 and 1.5×10^3 Da in the whole CH. From the profile of the CH and those of its fractions (CH-1, CH-2), it can be assumed that population corresponding to peak at 10^5 Da, absent from the anion exchange fractions, is mainly composed of neutral polysaccharides. DA-1 (2.7×10^5 Da-86% w/w; 1.8×10^3 Da) and DA-2 (4.2×10^5 Da-100% w/w) fractions were found to contain polysaccharides with relatively smaller molecular weights. The two main abundant peaks in DA-1 and DA-2 fractions are both originated from the main population identified the whole DA at 3.2×10^5 Da (Figures 4.2 and 4.4). The minor low-molecular weight fraction of DA-1 may be due to the degradation of the polysaccharides and/or to the dissociation of non-covalently bound oligosaccharides during the purification and dialysis steps.

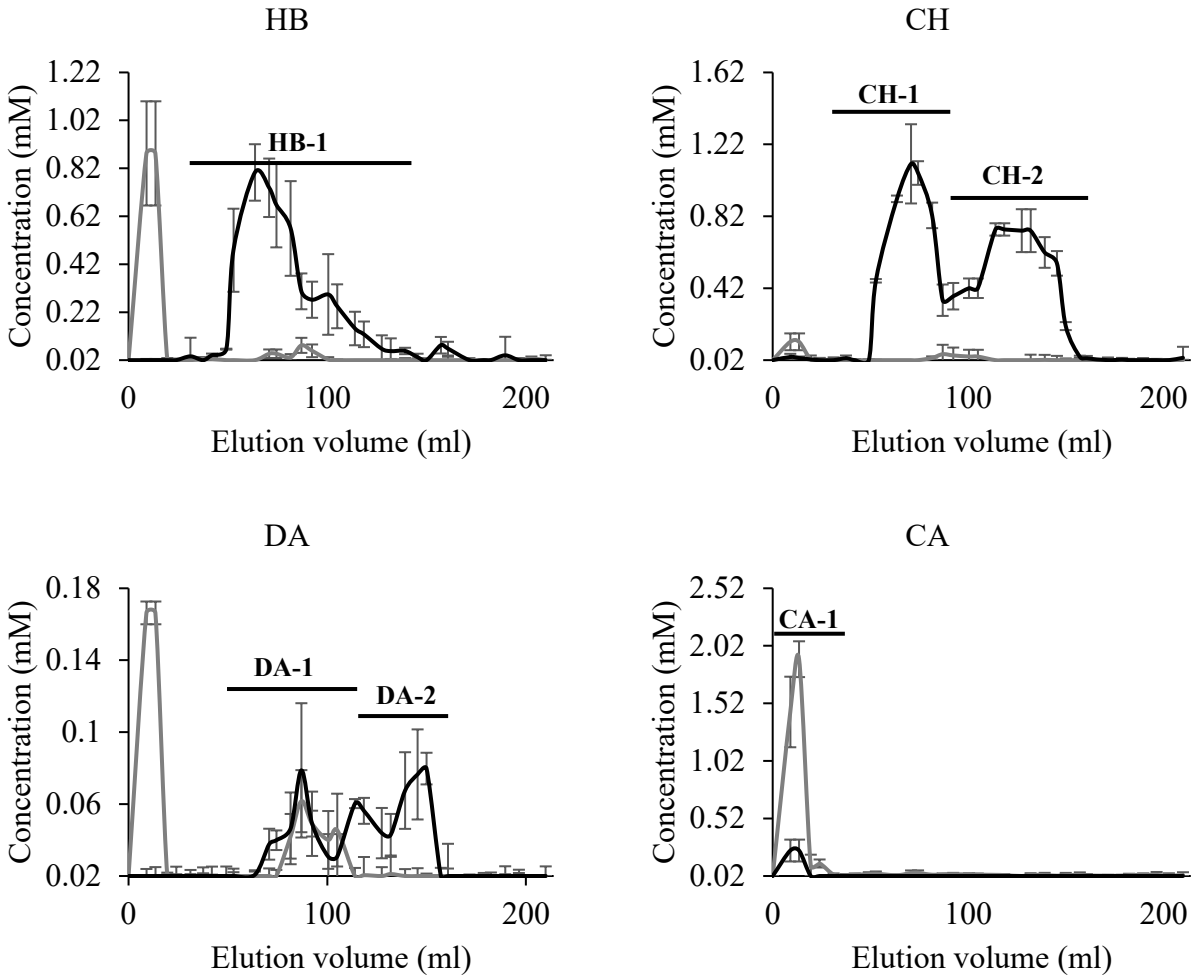

Figure 4.3 Uronic acid and neutral sugars contents of the fractions obtained by anion exchange chromatography of the extracts. HB: hot buffer extract; CH: chelating agents extract; DA: diluted alkali extract; CA: concentrated alkali extract. Black line: uronic acids; grey line: neutral sugars.

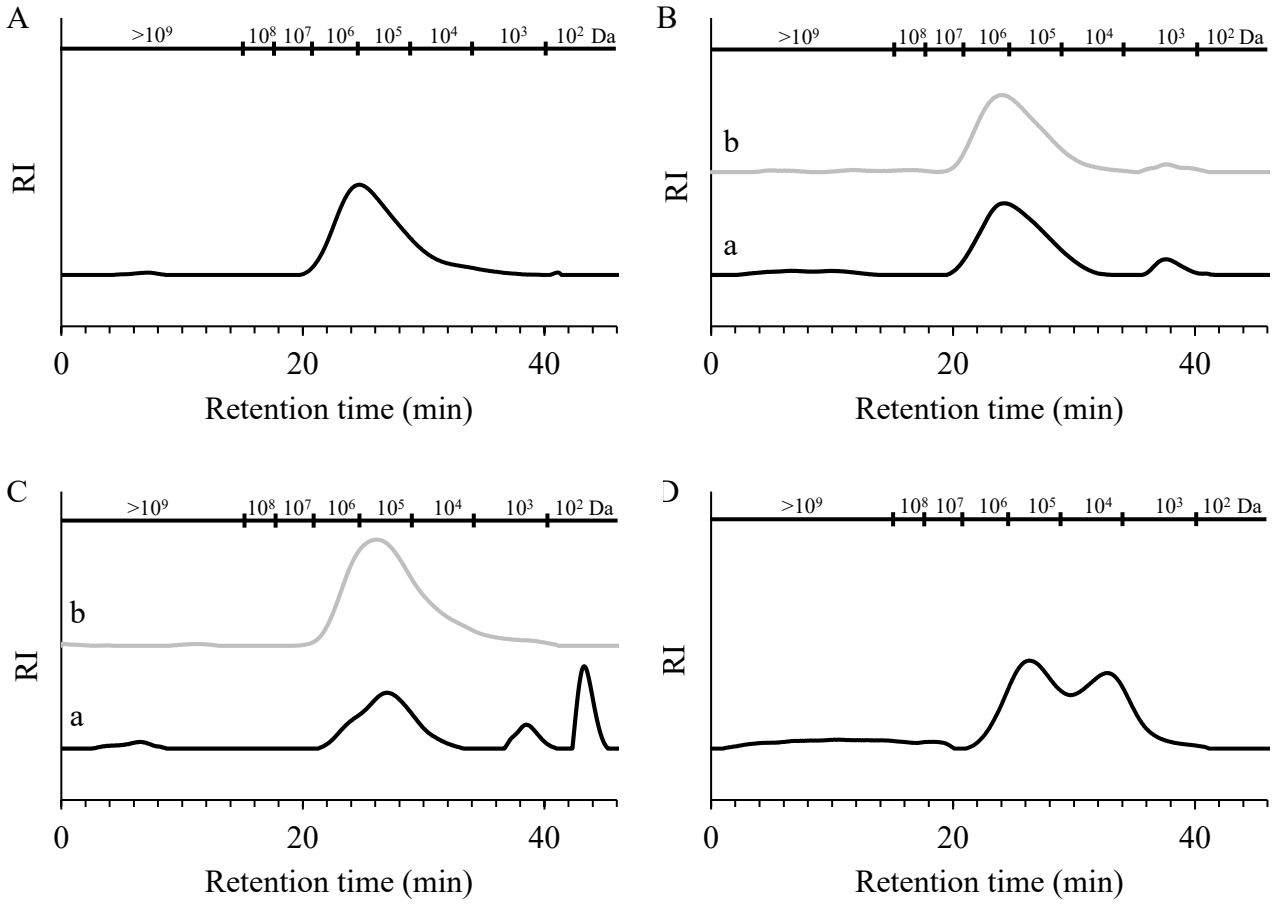

Figure 4.4 Molecular weight distribution in the anion exchange fractions obtained from extracts. A: Hot buffer extract fraction 1 (HB-1); B: Chelating agents extract fraction 1 (CH-1) (a), fraction 2 (CH-2) (b); C: Diluted alkali extract fraction 1 (DA-1) (a), fraction 2 (DA-2) (b); D: Concentrated alkali extract fraction 1 (CA-1).

In CA-1 fraction, two peaks were identified, one at 3.9×10^5 Da (53% w/w) and the other at 1.8×10^4 Da as for the whole CA. It can be hypothesized that CA is composed of two polysaccharide populations exhibiting high intermolecular interactions.

The monosaccharide profiles (Table 4.3) show that HB-1, CH-1 and CH-2 were mainly composed of UA residues (>90%, mol%), indicating a prevalence of homogalacturonan. Among the fractions with high proportions of pectic polysaccharides, the ratio of Ara and Gal to Rha provides an estimate of the level of branching of RGI. The fractions from DA had the highest ratios, indicating the presence of longer or more abundant arabinan and galactan side chains. The abundance of RGI was estimated from the proportion of Ara, Gal and Rha, taking into consideration the backbone of RGI includes equal moles of Rha and GalA. HB-1 contained 8.2% mol% of RGI, while the proportion in CH-1 and CH-2 fractions were 5.1 and 4.7%, with limited branching (1.7:1 and 4.2:1, respectively). While UA residues were also present in DA-1 (57.7%) and DA-2 (69.7%), these fractions contained higher amounts of neutral sugars, indicating the presence of more complex structures. From the Rha molar proportions (3.0 and 2.5%), it can be assumed that RGI is more abundant in DA-1 and DA-2 than in the other fractions. RGI in DA-1 contained more or longer arabinan and galactan branches (11.5:1) than that in DA-2 (10.0:1). CA-1 fraction was slightly enriched in UA compared to the whole CA, but it was still primarily composed of Glc, Xyl and Man, confirming the prevalence of non-pectic polysaccharides.

4.4.5. Sugar linkage analysis

To gain information on the structures and the distribution of the polysaccharides in the cranberry extracts, sugar linkages were analyzed (Table 4.4). The sugar linkages adjacent to GalA residues are usually difficult to hydrolyze, so they were not detected as PMAAs. A carboxyl reduction step was carried out to convert GalA residues to neutral Gal, followed by formation of PMAAs via methylation. By comparing the relative abundance of Gal's PMAAs with and without pre-reduction, the linkage information related to GalA could be obtained. The relatively low solubility of polysaccharides extracts samples in DMSO before methylation (Hilz et al., 2005) was corrected by considering the complete monosaccharide profile obtained upon chemical hydrolysis of polysaccharides into monosaccharides.

Table 4.3 Monosaccharide profile of fractions from anion exchange chromatography.

Frac.	Yield %[a]	Monosaccharide profile (mol%)						Pectic neutral sugars %[c]	Branching[d]
		Rha	Ara	Gal	Glc	Man + Xyl	UA[b]		
HB-1	70	1.4 ± 0.1^{g}	1.0 ± 0.1^{i}	4.5 ± 0.9^{f}	0.9 ± 0.3^{f}	1.3 ± 0.4^{g}	90.9 ± 5.1^{e}	75.6	4.0
CH-1	47	1.4 ± 0.3^{fg}	1.9 ± 1.1^{hi}	0.4 ± 0.0^{h}	0.2 ± 0.0^{g}	0.9 ± 0.4^{g}	95.2 ± 8.6^{e}	77.7	1.7
CH-2	49	0.8 ± 0.2^{h}	2.8 ± 0.4^{h}	0.4 ± 0.1^{h}	0.3 ± 0.1^{g}	0.5 ± 0.3^{g}	95.2 ± 13.3^{e}	82.8	4.2
DA-1	44	3.0 ± 0.5^{e}	27.8 ± 2.9^{e}	6.5 ± 1.5^{ef}	2.3 ± 1.4^{f}	2.8 ± 0.5^{f}	57.7 ± 3.9^{f}	88.0	11.5
DA-2	23	2.5 ± 0.1^{e}	22.9 ± 0.1^{f}	2.1 ± 0.7^{g}	1.2 ± 0.5^{f}	1.6 ± 0.2^{g}	69.7 ± 1.2^{ef}	90.9	10.0
CA-1	84	2.1 ± 0.5^{ef}	7.6 ± 2.2^{g}	8.9 ± 1.1^{e}	48.8 ± 0.9^{e}	19.2 ± 0.2^{e}	13.4 ± 0.0^{g}	21.4	7.7

All values are expressed as mean ± SD.

HB: hot buffer extract; CH: chelating agents extract; DA: diluted alkali extract; CA: concentrated alkali extract; L-Rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-xylose (Xyl), Uronic acids (UA) and D-mannose (Man).

a Determined by colorimetric method as percentage of total sugars.

b Determined by m-hydroxydiphenyl colorimetric method.

c Determined as (Rhamnose + Arabinose + Galactose)/sum of neutral sugars.

d Determined as (Arabinose + Galactose)/Rhamnose.

e-h Within the same column, means with different letters are significantly different at $P \leq 0.05$.

Table 4.4 Sugar linkage composition (% mol/mol) of cranberry extracts as determined by GC-MS.

	HB	CH	DA	CA
1,2,4-Rha*p*	**0.2**	**0.2**	**0.4**	**0.0**
T-Ara*f*	1.4	1.2	2.3	0.0
1,3-Ara*f*	0.2	0.2	0.4	0.0
1,5-Ara*f*	7.0	4.9	7.8	29.2
1,2,5-Ara*f*	0.1	0.1	0.2	0.0
1,3,5-Ara*f*	0.4	0.3	1.0	0.0
Tot Ara	**9.0**	**6.6**	**11.7**	**29.2**
T-Gal*p*	0.3	0.0	0.4	0.0
1,2-Gal*p*	0.1	0.3	0.1	1.0
1,3-Gal*p*	0.4	0.3	0.6	0.9
1,4-Gal*p*	0.0	0.0	0.7	0.0
1,6-Gal*p*	<0.1	0.0	<0.1	0.0
1,3,4-Gal*p*	0.0	0.0	0.0	0.0
1,3,6-Gal*p*	<0.1	<0.1	0.1	0.0
1,2,3,4,6-Gal*p*	0.1	<0.1	<0.1	<0.1
Tot Gal	**1.0**	**0.7**	**2.0**	**1.9**
T-Glc*p*	7.3	3.4	4.2	29.5
1,2-Glc*p*	0.1	0.0	0.0	0.4
1,4-Glc*p*	2.2	1.8	1.8	4.1
1,4,6-Glc*p*	0.2	0.3	0.2	0.2
1,2,3,4,6-Glc*p*	0.0	0.0	<0.1	<0.1
Tot Glc	**9.7**	**5.5**	**6.2**	**34.2**
T-Xyl*p*	**0.2**	**0.3**	**0.4**	**0.3**

	HB	CH	DA	CA
T-Man*p*	7.1	3.3	4.1	28.8
1,2,3,4,6-Man*p*	0.1	<0.1	<0.1	0.1
Tot Man	**7.2**	**3.4**	**4.1**	**28.9**
T-Gal*p*A	2.0	1.9	0.0	0.0
1,2-Gal*p*A	0.0	0.0	2.0	0.0
1,3-Gal*p*A	10.3	0.6	0.0	0.0
1,4-GalpA	57.6	78.2	68.4	0.0
1,6-Gal*p*A	0.0	0.1	0.3	0.0
1,2,4-Gal*p*A	0.0	0.5	0.7	0.0
1,3,4-Gal*p*A	0.5	0.5	1.1	0.0
1,3,6-Gal*p*A	0.5	0.7	0.7	0.0
1,4,6-Gal*p*A	1.9	0.9	1.9	0.0
Tot GalA	**72.8**	**83.4**	**75.2**	**5.5**
Arabinan chain length[a]	8.8	7.5	9.7	-
Arabinan branching[b]	0.5	0.5	1.2	-
Galactan/AGI chain length[c]	1.1	-	4.7	-
Galactan/AGI branching[d]	0.1	-	0.2	-
AGII chain length[e]	3.0	-	5.1	-
AGII branching[f]	0.1	-	0.7	-
RGI %[g]	0.6	0.4	1.1	-

HB: hot buffer extract; CH: chelating agents extract; DA: diluted alkali extract; CA: concentrated alkali extract.
[a] Calculated as 1+(1,5-Ara*f* + 1,2,5-Ara*f* + 1,3,5-Ara*f*)/(T-Ara*f* - 1,2,5-Ara*f* - 1,3,5-Ara*f*).
[b] Calculated as (1,2,5-Ara*f* + 1,3,5-Ara*f*)/(T-Ara*f* - 1,2,5-Ara*f* - 1,3,5-Ara*f*).
[c] Calculated as 1+(1,4-Gal*p* + 1,3,4-Gal*p*)/(T-Gal*p* - 1,3,4-Gal*p* - 1,3,6-Gal*p*).
[d] Calculated as (1,3,4-Gal*p*)/(T-Gal*p* - 1,3,4-Gal*p* - 1,3,6-Gal*p*).
[e] Calculated as 1+(1,3-Gal*p* + 1,3,6-Gal*p*)/(T-Gal*p* - 1,3,4-Gal*p* - 1,3,6-Gal*p*).
[f] Calculated as (1,3,6-Gal*p*)/(T-Gal*p* - 1,3,4-Gal*p* - 1,3,6-Gal*p*).
[g] Calculated as 2 × 1,2,4-Rha*p*/(1,4-Gal*p*A + 1,2,4-Rha*p* + 1,2,4-Gal*p*A + 1,3,4-Gal*p*A).

Chain length can be estimated from the ratio of twice and thrice-substituted residues to terminal residues, combined with general notions about conserved chain characteristics found across higher plants (Mohnen, 2008), while the degree of branching is calculated as the number of thrice substituted (branching-point) residues per branch.

Arabinan chains were found in all extracts. In cranberry HB, the arabinan chains appeared to have an average length of 8.8 residues bound by 1,5 linkage, of which 0.5 residues per chain possessed branching at C2 or C3. In CH and DA, these chains appeared to have a length of 7.5 and 9.7 residues, respectively, with 0.5 and 1.2 branched residues per chain. In CA, the only form of Ara detected was 1,5-Ara*f*, hence it was only possible to infer the presence of arabinan.

Galactan and type I arabinogalactan (AGI) are known to possess a main chain composed by β-(1,4) linked galactopyranose residues (Yahia et al., 2019). Low amounts of terminal Gal were only found in HB and DA. In HB, very short chains with a calculated average length of 1.1 residues were detected. Since only terminal and 1,3,4 branched Gal residues were observed in samples without pre-reduction, the presence of monomeric Gal and less common trimeric galactan chains was hypothesized to be present. On the other hand, in DA the 1,4-Gal chains appeared to be longer (4.7 residues), while the degree of branching was similar to what observed in HB (0.2 branched residues per chain, compared to 0.1). After carboxyl reduction, an increase in relative abundance of 1,4-Gal linkage was observed in all extracts except CA, indicating the presence of 1,4-GalA (characteristic linkage of homogalacturonan) in HB, CH and DA.

Type II arabinogalactan (AGII) is characterized by a main chain of β-(1,3) linked galactopyranose residues (Heredia et al., 1995). This linkage was found in all extracts: for CH and CA, an average length of the chains could not be calculated, as no terminal Gal residue was observed, while the average length appeared to be 3.0 residues in HB, and 5.1 in DA.

Some branching, represented by 1,3,6-Gal*p*, was also observed (0.1 and 0.7 branched residues per chain in HB and DA, respectively). Coherently with the monosaccharide profile obtained by HPSEC, Gal residues were less abundant than Ara in HB, CH and DA. Xylopyranose was found exclusively as terminal, non-reducing residues in all extracts. This indicated that they didn't originate from xylans or other Xyl-including hemicelluloses where Xyl is found as polymeric chains, but more likely from Xyl decorations, such as those found on xyloglucan and xylogalacturonan (Mohnen, 2008).

The presence of 1,2,4-Gal*p*A and 1,3,4-Gal*p*A can provide information on the abundance of acetyl esterification and decoration of galacturonic acid residues, as seen in xylogalacturonan. These were found in HB, CH and DA at ratios of 0.9, 1.3 and 2.5 residues every 100 1,4-Gal*p*A residues, respectively.

1,2,4-Rha*p* represented the only type of rhamnopyranose residue detected, indicating that RGI was highly branched and confirming the absence of detectable levels of the other Rha-containing polysaccharide (rhamnogalacturonan II) (Mohnen, 2008).

Considering the backbone of RGI as an alternance of galacturonic acid and Rha residues, the percentage of galacturonic acid-containing polymers represented by it was estimated to be 0.6% in HB, 0.4% in CH and 1.1% in DA. Despite the limitations due to the partial resistance to hydrolysis of the polysaccharide, these percentages were expected to reflect the abundance of hairy regions in the pectins, as they are composed by the neutral sidechains attached to RGI backbone. It is worth noting that, among the three extracts where this ratio of RGI to homogalacturonan could be calculated, the lowest value was from CH, confirming that it contained more linear pectic polysaccharides. Finally, the presence of high amounts of terminal non-reducing Glc, 1,4-Glc*p* and 1,4,6-Glc*p* was indicative of cellulose and hemicelluloses, common components of cell wall in lignified tissues (Bajpai, 2018).

4.4.6. Structural characterisation by NMR

CH and CA extracts were characterized by 2D and 3D NMR spectroscopy. The chemical shifts, reported in Table 4.5, were attributed to glycosidic residues by comparison with published values and with standards of known composition. The HSQC spectrum of CH (Figure 4.5A) displayed intense signals of 1,4-α-Gal*p*A residues (both methyl esterified and not), 1,5-α-Ara*f*, 1,3-α-Ara*f*, terminal non-reducing α- and β-Ara*f*, β-Gal*p* and α-Gal*p*A, and GalA reducing ends (4-α-Gal*p*A and 4-β-Gal*p*A). These linkages indicate the predominance of homogalacturonan and pectic arabinan structures in the extract. The ratio of 3.0:1 between the intensity of non-methylated and methylated 1,4-α-Gal*p*A indicated the prevalence of the first. The spectrum also contained lower-intensity signals attributable to galactan structures (1,4-β-Gal*p*) arabinogalactan type II (1,3,6-β-Gal*p*) and arabinan branching (1,2,3,5-α-Ara*f*).

Table 4.5 Chemical shift assignments of chelating agents (CH) and concentrated alkali (CA) extracts, referenced to DSS.

CH	C1	H1	C2	H2	C3	H3	C4	H4	C5	H5
1,4-α-Gal*p*A	101.6	5.07	70.9	3.74	71.7	3.97	80.8	4.40	74.1	4.69
1,5-α-Ara*f*	110.2	5.08	83.6	4.12	79.4	4.00	85.0	4.19	69.6	3.88, 3.75
1,4-α-Gal*p*A	101.9	5.11								
1,4-α-Gal*p*A6Me	102.6	4.90	71.5	3.95	70.9	3.72				
1,5-α-Ara*f*	110.3	5.10								
1,3-α-Ara*f*	109.8	5.17	84.0	4.13	86.8	3.97	82.4	4.35		
1,4-α-Gal*p*A6Me	102.8	4.95								
T-β-Ara*f*	104.1	5.08	77.1	4.03	84.8	3.89	79.2	4.12	65.8	3.79, 3.71
4-β-Gal*p*A	98.8	4.58	74.3	3.48	75.1	3.73	80.8	4.36		
1,3-α-Ara*f*	109.8	5.14								
T-α-Ara*f*	111.8	5.24	84.0	4.20						
1,2,3,5-α-Ara*f*	109.1	5.23	88.0	4.29	83.1	4.23				
4-α-Gal*p*A	94.9	5.30	71.5	3.98	80.1	4.40	73.4	4.49		
T-α-Ara*f*	111.8	5.16	83.9	4.19						
1,3,6-β-Gal*p*	105.8	4.52	72.2	3.66	82.8	3.72	71.1	4.12		
1,4-β-Gal*p*	107.1	4.62	76.0	3.75	74.5	3.66				
CA	C1	H1	C2	H2	C3	H3	C4	H4	C5	H5
1,4-β-Glc*p*	105.0	4.52	76.0	3.37	76.2	3.81	81.6	3.67		
T-α-Ara*f*	111.8	5.16	83.9	4.19	79.2	3.94	86.5	4.07	64.0	3.82, 3.71
1,4-β-Man*p*	102.6	4.75	79.1	3.83	72.8	4.11	74.2	3.78	77.1	3.56
1,4-β-Man*p*2OAc*	101.2	5.06	74.7	3.84	81.4	3.56	72.2	3.66	63.9	3.72, 3.55
1,5-α-Ara*f*	110.2	5.07	83.5	4.13	79.3	4.00	85.4	4.20	69.7	3.88, 3.79
T-α-Xyl*p*	101.5	4.93	74.2	3.55	75.8	3.72	72.2	3.64	64.2	3.71, 3.58
T-β-Gal*p*	107.2	4.55	71.4	3.92	76.0	3.67	73.8	3.62		
1,4-β-Man*p*2OAc*	101.1	5.10	75.0	3.85	72.2	3.66	83.0	3.60	74.2	3.55
? -α-Glc*p**	101.4	5.01	72.2	3.93	71.2	3.82	72.2	4.00		
1,5-α-Ara*f*	110.2	5.10								
1,4-β-Man*p*2OAc*	101.2	5.13		3.66						
T-α-Ara*f*	111.8	5.24	84.0	4.22						
T-β-Glc*p*A*	103.9	4.62	75.0	3.64	79.3	3.80	78.5	3.46		3.51
1,3-α-Gal*p*A*	101.3	5.20	70.9	4.14	80.2	3.94	72.1	4.14		
1,4-β-Gal*p*	107.0	4.61	76.0	3.76						
1,3-α-Ara*f*	109.8	5.17								
1,3-α-Ara*f*	109.8	5.14	82.6	4.35	86.4	3.96	83.9	4.13	63.5	3.80
1,4-β-Man*p*	102.8	4.73								

?: undetermined linkage; *:tentative assignment, on the basis of database match.

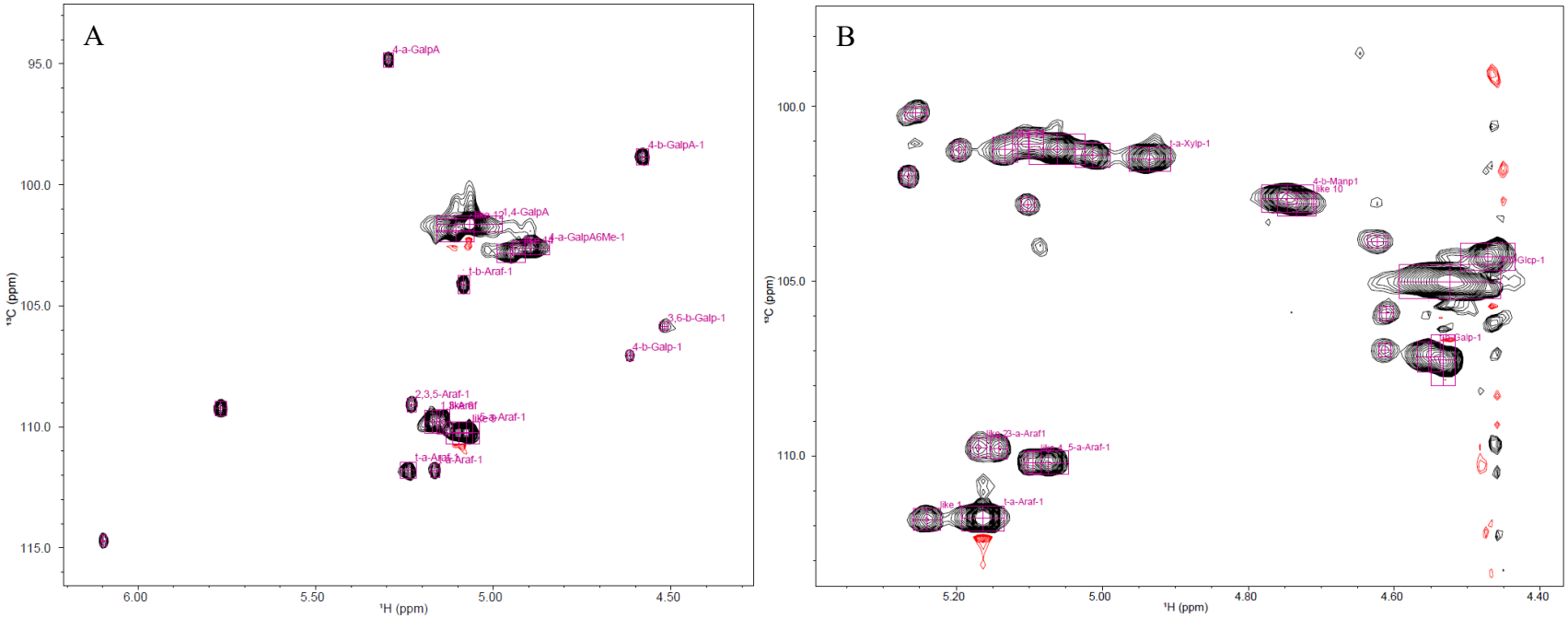

Figure 4.5 Selected region of $^{1}H,^{13}C$ HSQC spectra. A: Chelating agents extract; B: concentrated alkali extract.

The ratio of the integral intensities of anomeric hydrogen (H1) of arabinan-associated residues in the spectrum was T-Ara*f*:1,5-α-Ara*f*:1,3-α-Ara*f*:1,2,3,5-α-Ara*f* = 5.1:24.6:8.2:1.2. Arabinan appeared, therefore, to possess 1,5-linked backbone (63% of Ara residues) and branches with 1,3-linkage (21%). A similar structure was observed in sugar beet arabinan (Caffall & Mohnen, 2009; Sakamoto & Kawasaki, 2003). Since branching points (1,2,3,5-α-Ara*f*) represent only 3% of the detected Ara residues, the pectic arabinan may possess few, long 1,3-linked branches and monomeric 1,2-linked Ara*f* decorations. Using the same equations applied on the GC-MS results to estimate arabinan length and branching, and taking into consideration that to one mole of 1,2,3,5-α-Ara*f* correspond two of non-backbone terminal Ara residues, average length of the 1,5-linked arabinan structures results 11.0 residues, while branching is 0.5. The lack of detectable Rha residues further suggested the presence of long pectic arabinan chains, attached to very short rhamnogalacturonan I backbones.

The spectrum from CA (Figure 4.5B) showed, in addition to terminal and 1,5-linked α-Ara*f* signals, very intense signals of 1,4-β-Glc*p* and 1,4-β-Man*p* (including some Man tentatively identified as acetyl esterified in C2), as well as terminal β-Gal*p* and α-Xyl*p*. This fraction is therefore characterized by the presence of abundant glucomannan, as well as some pectic arabinan. Less abundant residues include more pectic galactan and arabinan fragments (1,3-α-Ara*f* and 1,4-β-Gal*p*), and the tentatively attributed terminal β-Glc*p*A (possibly deriving from a trace of glucuronoxylan) and 1,3-α-Gal*p*A. Estimated chain length of pectic arabinan and galactan, calculated from the proportion of anomeric hydrogen peaks intensity, were 1.5, 1.2 respectively. It should be noted that, while some of the terminal Ara and Gal may actually be decorations of hemicellulosic polymers such as xyloglucan, the absence of detectable branched Glc residues (such as 1,4,6-β-Glc*p*) makes the possible relevance of these structures on the estimate less significant. As no terminal Glc or Man residues could be clearly detected, length of glucomannan could not be estimated.

4.5. Conclusions

AIS isolated from Stevens variety cranberries pomace represented 80% of the dry pomace weight -a value in the range of those reported for varieties of apple, citrus and black currant pomaces- and were rich in GalA. Aqueous extracts obtained from AIS contained abundant pectic polysaccharides, except the concentrated alkali one. Chelating agent extract had the highest yield of poly-and oligosaccharides, as well as the highest proportion of GalA.

Liquid chromatography techniques and monosaccharide linkage analysis completed the characterization of the extracts to a level of detail unprecedented for the cell wall of fruit. The obtained information, although it did not account for the possible variations present in different cranberry batches due to cultivation and storage conditions, is valuable in view of a possible utilization of cranberry pomace as source of polysaccharides, and for the optimization of methods for isolating specific pectic polysaccharide sub-fractions to be used as functional food ingredients.

4.6. References

Abeywickrama, G., Debnath, S. C., Ambigaipalan, P., & Shahidi, F. (2016). "Phenolics of Selected Cranberry Genotypes (Vaccinium macrocarpon Ait.) and Their Antioxidant Efficacy". Journal of Agricultural and Food Chemistry *64*(49): 9342–9351.

Anumula, K. R., & Taylor, P. B. (1992). "A comprehensive procedure for preparation of partially methylated alditol acetates from glycoprotein carbohydrates". Analytical Biochemistry *203*(1): 101–108.

Aura, A.-M., Holopainen-Mantila, U., Sibakov, J., Kössö, T., Mokkila, M., & Kaisa, P. (2015). "Bilberry and bilberry press cake as sources of dietary fibre". Food & Nutrition Research *59*(1): 28367.

Bajpai, P. (2018). "Wood and Fiber Fundamentals". In *Biermann's Handbook of Pulp and Paper* (pp. 19–74). Elsevier.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). "New method for quantitative determination of uronic acids". Analytical Biochemistry *54*(2): 484–489.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding". Analytical Biochemistry *72*(1–2): 248–254.

Caffall, K. H., & Mohnen, D. (2009). "The structure, function, and biosynthesis of plant cell wall pectic polysaccharides". Carbohydrate Research *344*(14): 1879–1900.

Christiaens, S., Van Buggenhout, S., Chaula, D., Moelants, K., David, C. C., Hofkens, J., … Hendrickx, M. E. (2012). "In situ pectin engineering as a tool to tailor the consistency and syneresis of carrot purée". Food Chemistry *133*(1): 146–155.

Clague, J. A., & Fellers, C. R. (1934). "Relation of Benzoic Acid Content and Other Constituents of Cranberries to Keeping Quality.". Plant Physiology *9*(3): 631–6.

Coimbra, M. A., Waldron, K. W., & Selvendran, R. R. (1994). "Isolation and characterisation of cell wall polymers from olive pulp (Olea europaea L.)". Carbohydrate Research *252*: 245–262.

Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., & Bax, A. (1995). "NMRPipe: A multidimensional spectral processing system based on UNIX pipes". Journal of Biomolecular NMR *6*(3): 277–293.

Deng, J., Shi, Z.-J., Li, X.-Z., & Liu, H.-M. (2013). "Soluble polysaccharides isolation and characterization from rabbiteye blueberry (Vaccinium ashei) fruits". BioResources *8*(1): 405–419.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). "Colorimetric Method for Determination of Sugars and Related Substances". Analytical Chemistry *28*(3): 350–356.

Elwell, W. E., & Dehn, W. M. (1939). "Pectic content of plant materials". Plant Physiology *4*: 809–816.

Fan, H., Mazza, G., & Liao, X. (2010). "Purification, composition and antioxidant activity of polysaccharides from wolfberry, cherry, kiwi and cranberry fruits". Croatian Journal of Food Science Technology *2*(1): 9–17.

Harris, P. J., Henry, R. J., Blakeney, A. B., & Stone, B. A. (1984). "An improved procedure for the

methylation analysis of oligosaccharides and polysaccharides". Carbohydrate Research *127*(1): 59–73.
Harrison, J. E., Oomah, B. D., Diarra, M. S., & Ibarra-Alvarado, C. (2013). "Bioactivities of pilot-scale extracted cranberry juice and pomace". Journal of Food Processing and Preservation *37*(4): 356–365.
Heredia, A., Jiménez, A., & Guillén, R. (1995). "Composition of plant cell walls". Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung *200*(1): 24–31.
Hilz, H., Bakx, E. J., Schols, H. A., & Voragen, A. G. J. (2005). "Cell wall polysaccharides in black currants and bilberries—characterisation in berries, juice, and press cake". Carbohydrate Polymers *59*(4): 477–488.
Hilz, H., Williams, P., Doco, T., Schols, H. A., & Voragen, A. G. J. (2006). "The pectic polysaccharide rhamnogalacturonan II is present as a dimer in pectic populations of bilberries and black currants in muro and in juice". Carbohydrate Polymers *65*(4): 521–528.
Huisman, M. M. ., Schols, H. ., & Voragen, A. G. . (1998). "Cell wall polysaccharides from soybean (Glycine max.) meal. Isolation and characterisation". Carbohydrate Polymers *37*(1): 87–95.
Johnson, B. A., & Blevins, R. A. (1994). "NMR View: A computer program for the visualization and analysis of NMR data". Journal of Biomolecular NMR *4*(5): 603–614.
Kaya, M., Sousa, A. G., Crépeau, M.-J., Sørensen, S. O., & Ralet, M.-C. (2014). "Characterization of citrus pectin samples extracted under different conditions: influence of acid type and pH of extraction". Annals of Botany *114*(6): 1319–1326.
Khodaei, N., & Karboune, S. (2013). "Extraction and structural characterisation of rhamnogalacturonan I-type pectic polysaccharides from potato cell wall". Food Chemistry *139*(1–4): 617–623.
Kolanowski, W., Laufenberg, G., & Kunz, B. (2004). "Fish oil stabilisation by microencapsulation with modified cellulose.". International Journal of Food Sciences and Nutrition *55*(4): 333–43.
Kosmala, M., Kołodziejczyk, K., Markowski, J., Mieszczakowska, M., Ginies, C., & Renard, C. M. G. C. (2010). "Co-products of black-currant and apple juice production: Hydration properties and polysaccharide composition". LWT - Food Science and Technology *43*(1): 173–180.
Ma, Y., Luo, J., & Xu, Y. (2019). "Co-preparation of pectin and cellulose from apple pomace by a sequential process". Journal of Food Science and Technology *56*(9): 4091–4100.
Makarova, E. N., Shakhmatov, E. G., & Belyy, V. A. (2018). "Structural studies of water-extractable pectic polysaccharides and arabinogalactan proteins from Picea abies greenery". Carbohydrate Polymers.
Masli, M. D. P., Rasco, B. A., & Ganjyal, G. M. (2018). "Composition and Physicochemical Characterization of Fiber-Rich Food Processing Byproducts". Journal of Food Science *83*(4): 956–965.
Mohnen, D. (2008). "Pectin structure and biosynthesis.". Current Opinion in Plant Biology *11*(3): 266–277.
Narwojsz, A., Tańska, M., Mazur, B., & Borowska, E. J. (2019). "Fruit Physical Features, Phenolic Compounds Profile and Inhibition Activities of Cranberry Cultivars (Vaccinium macrocarpon) Compared to Wild-Grown Cranberry (Vaccinium oxycoccus)". Plant Foods for Human Nutrition *74*(3): 300–306.
Popper, Z. A., & Fry, S. C. (2008). "Xyloglucan-pectin linkages are formed intra-protoplasmically, contribute to wall-assembly, and remain stable in the cell wall". Planta *227*(4): 781–794.
Prabasari, I., Pettolino, F., Liao, M.-L., & Bacic, A. (2011). "Pectic polysaccharides from mature orange (Citrus sinensis) fruit albedo cell walls: Sequential extraction and chemical characterization". Carbohydrate Polymers *84*(1): 484–494.

Ralet, M.-C., Crépeau, M.-J., Buchholt, H.-C., & Thibault, J.-F. (2003). "Polyelectrolyte behaviour and calcium binding properties of sugar beet pectins differing in their degrees of methylation and acetylation". Biochemical Engineering Journal *16*(2): 191–201.

Renard, C. M. G. C., & Thibault, J.-F. (1993). "Structure and properties of apple and sugar-beet pectins extracted by chelating agents". Carbohydrate Research *244*(1): 99–114.

Renard, C. M. G. C., Voragen, A. G. J., Thibault, J. F., & Pilnik, W. (1990). "Studies on apple protopectin: I. Extraction of insoluble pectin by chemical means". Carbohydrate Polymers *12*(1): 9–25.

Sakamoto, T., & Kawasaki, H. (2003). "Purification and properties of two type-B α-l-arabinofuranosidases produced by Penicillium chrysogenum". Biochimica et Biophysica Acta (BBA) - General Subjects *1621*(2): 204–210.

Shakhmatov, E. G., Makarova, E. N., & Belyy, V. A. (2019). "Structural studies of biologically active pectin-containing polysaccharides of pomegranate Punica granatum". International Journal of Biological Macromolecules.

Singh, I., Gautam, L. K., & Kaur, I. R. (2016). "Effect of oral cranberry extract (standardized proanthocyanidin-A) in patients with recurrent UTI by pathogenic E. coli: a randomized placebo-controlled clinical research study". International Urology and Nephrology *48*(9): 1379–1386.

Taylor, R. L., & Conrad, H. E. (1972). "Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl group". Biochemistry *11*(8): 1383–1388.

Toukach, P. V., & Egorova, K. S. (2016). "Carbohydrate structure database merged from bacterial, archaeal, plant and fungal parts". Nucleic Acids Research.

United States Department of Agriculture, & Agricultural Research Service. (2019). *Full Report (All Nutrients): 09078, Cranberries, raw*.

Vattem, D. A., & Shetty, K. (2002). "SOLID-STATE PRODUCTION OF PHENOLIC ANTIOXIDANTS FROM CRANBERRY POMACE BY RHIZOPUS OLIGOSPORUS". Food Biotechnology *16*(3): 189–210.

Vierhuis, E., Schols, H. A., Beldman, G., & Voragen, A. G. J. (2000). "Isolation and characterisation of cell wall material from olive fruit (Olea europaea cv koroneiki) at different ripening stages". Carbohydrate Polymers *43*(1): 11–21.

Villanueva-Suárez, M. J., Pérez-Cózar, M. L., & Redondo-Cuenca, A. (2013). "Sequential extraction of polysaccharides from enzymatically hydrolyzed okara byproduct: Physicochemical properties and in vitro fermentability". Food Chemistry *141*(2): 1114–1119.

White, B. L., Howard, L. R., & Prior, R. L. (2010). "Proximate and Polyphenolic Characterization of Cranberry Pomace". Journal of Agricultural and Food Chemistry *58*(7): 4030–4036.

Yahia, E. M., Carrillo-López, A., & Bello-Perez, L. A. (2019). "Carbohydrates". In *Postharvest Physiology and Biochemistry of Fruits and Vegetables* (pp. 175–205). Elsevier.

Zbikowska, A., Kowalska, M., & Pieniowska, J. (2018). "Assessment of shortcrust biscuits with reduced fat content of microcrystalline cellulose and psyllium as fat replacements". Journal of Food Processing and Preservation *42*(8): e13675.

Zhang, W., Fan, X., Gu, X., Gong, S., Wu, J., Wang, Z., … Wang, S. (2020). "Emulsifying properties of pectic polysaccharides obtained by sequential extraction from black tomato pomace". Food Hydrocolloids *100*: 105454.

Zou, Y. F., Zhang, Y. Y., Paulsen, B. S., Rise, F., Chen, Z. L., Jia, R. Y., … Yin, Z. Q. (2020). "Structural features of pectic polysaccharides from stems of two species of Radix Codonopsis and their antioxidant activities". International Journal of Biological Macromolecules.

CONNECTING STATEMENT 3

The analysis of cranberry cell wall polysaccharides reported in chapter IV revealed the presence of abundant pectic polysaccharides with different chemical characteristics in three of the four extracts. To characterize the structural features of pectic polysaccharides, a more focused study of the composition and structure of purified pectic polysaccharide fractions was designed. The study, presented in chapter V, focused on the treatment of selected pectic polysaccharides with a series of pure pectin-degrading enzymatic activities and on the determination of composition and linkage of the liberated fragments. The substrate specificity of the enzymes allows the characterization of the relative abundance of the rhamnogalacturonan type I neutral branches, which are the most structurally complex parts of these polysaccharides and can yield the greatest diversity of oligosaccharides.

Spadoni Andreani, E. & Karboune, S. (2020). Characterization of the Structural Features of Cell Wall Pectic Polysaccharides in Stevens Variety Cranberry Pomace by Sequential Extraction Approach and Using Highly Specific Pectin-Hydrolyzing Enzymes. (*To be submitted*).

CHAPTER V. CHARACTERIZATION OF THE STRUCTURAL FEATURES OF CELL WALL PECTIC POLYSACCHARIDES IN STEVENS VARIETY CRANBERRY POMACE BY SEQUENTIAL EXTRACTION APPROACH AND USING HIGHLY SPECIFIC PECTIN-HYDROLYZING ENZYMES

5.1. Abstract

The potential of poly- and oligosaccharides as functional ingredients depends on the type and glycosidic linkages of their monosaccharide residues, which determine their techno-functional properties, their digestibility and their fermentability. To isolate the pectic polysaccharides of cranberry, alcohol insoluble solids were first obtained from pomace. A sequential extraction (hot phosphate buffer, chelating agents CH, diluted DA and concentrated sodium hydroxide) was then carried out. Pectic polysaccharides present in CH and DA extracts were purified by anion exchange and gel filtration chromatography, then sequentially exposed to commercially available pectin-degrading enzymes (pectin lyase, *endo*-polygalacturonase, *endo*-arabinanase/*endo*-galactanase/both). The composition and linkages of the generated fragments revealed important characteristic features, including the presence of homogalacturonan with varied methyl esterification extent, branched type I arabinogalactan and pectic galactan. The presence of arabinan with galactose branches was suggested upon the analysis of the fragments by LC-MS.

5.2. Introduction

Among plant cell wall polysaccharides, the pectic polysaccharides (PPS) are characterized by high versatility and complexity of their structure, composed of smooth and hairy regions (Kratchanova et al., 2010; Oosterveld et al., 1996). Indeed, the relevant chemical characteristics of PPS include smooth/hairy regions ratio, monosaccharide profile, amounts and distribution of substituents, molecular weight distribution and the typology of side chains they possess. The smooth region consists mainly of homogalacturonan and xylogalacturonan: the first is a linear polymer of α-(1,4)-D-galacturonic acid (Ridley et al., 2001), which is partially methyl esterified with at the carboxylic group of C6 and can be acetylated at C2 and C3. The second, generally less abundant in the cell wall, has the same backbone structure but presents β-xylopyranosyl monomers attached at the C3 of some of the galacturonic acid residues (Mohnen, 2008). Rhamnogalacturonan type I (RGI) is the main component of the hairy region, and has a conserved backbone, constituted by the repetition of the diglycosidic unit α-(1,4)-L-rhamnopyranose-α-(1,2)-D-galactopyranosyluronic acid (Bonnin et al., 2014). The galacturonic acid residues are partially acetylated as in homogalacturonan, while the rhamnose residues can be branched at C3 or C4 with oligomers of arabinose and galactose.

These neutral branches show great variability between different species, tissues and even in the same tissue in function of physiological changes such as ripening (Peña & Carpita, 2004; Yapo, 2011), but commonly include oligomers of α-(1,5)- L-arabinofuranose (arabinan), of β-(1→4)-D-galactopyranose (galactan), or mixed (arabinogalactans type I and II, which possess a backbone of galactan and arabinose-containing branches). Less common branches have also been reported, such as xylogalacturonan in apple cell wall (Oechslin et al., 2003; Schols et al., 1995), or arabinan with galactose-containing branches in sugar beet, potato, duckweed (Sakamoto & Sakai, 1995; Yu et al., 2015) among others. Rhamnogalacturonan type II (RGII) is a much less common but strongly conserved branched region, composed by the repetition of four branches containing neutral and acid glycosidic residues departing from a backbone of homogalacturonan (Vidal et al., 2000).

Smooth and hairy regions are found in variable proportions in extracted plant cell PPS and can be separated from each other only by enzymatic activity or strong chemical treatments. This indicates that they are covalently bound to each other, likely by the galacturonic acid extremities of their backbones (Bonnin et al., 2014; Mohnen, 2008). While the nature of the bonds between PPS and the other cell wall polymers is still unclear, it has been found that PPS are interconnected with both cellulose microfibrils (Wang et al., 2018) and xyloglucan (Popper & Fry, 2008), forming a complex network in the cell wall. Depending on their chemical structure, PPS can provide functionalities such as gelling, thickening and emulsion stabilizing (Bonnin et al., 2014). As one of the major constituents of soluble dietary fibers, PPS can reduce the risk of cardiovascular disease due to their effects on low density lipoproteins (Theuwissen & Mensink, 2008). Furthermore, PPS have been shown to possess *in vitro* prebiotic properties (Tingirikari, 2019) and have the potential to be utilized for the generation of pectic oligosaccharides with prebiotic (P. Li et al., 2016; Manderson et al., 2005; Olano-Martin et al., 2002) and other health-promoting properties such as antibacterial (S. Li et al., 2013) and antibiofilm effects (Sun et al., 2015).

Cranberry pomace, obtained during the juice manufacturing process, is a very abundant residual material. This material contains large amounts of cell wall polysaccharides, including PPS. Indeed, cranberry (*Vaccinium macrocarpon*) pomace has been recently studied as a source of antibiofilm oligosaccharides (Hotchkiss et al., 2015; Sun et al., 2015) derived from xyloglucan and arabinan, but the composition and linkage analyses were limited to the oligosaccharide fractions. The present study provides a first characterization of some of the PPS obtained from cranberry pomace by chelating agents (CH) and diluted alkaline (DA) extractions.

Selected PPS, isolated from the two extracts by anionic exchange and gel filtration chromatography, were analysed for their monosaccharide composition. Then, these polysaccharides were subjected to the sequential enzymatic degradation with highly specific pectin-degrading enzymatic activities to infer their structural properties from the liberated fragments (Holck et al., 2011; Olano-Martin et al., 2001). The contribution of our findings for the elucidation of the structural properties of RG I in cranberry cell walls was discussed.

5.3. Materials and methods

5.3.1. Materials

Stevens variety cranberries were provided by Atoka Cranberries Inc., Manseau, QC, Canada and stored at -20 °C. Enzymes were obtained from Megazyme, Bray, Ireland. Analytical grade reagents were from Sigma-Aldrich Co, St. Louis, MO, USA.

5.3.2. Preparation of alcohol insoluble solids

Pomace was obtained from frozen Stevens variety cranberries (Atoka Cranberries Inc.) by blending with a Vitamat commercial juicer (Rotor Lips AG, Uetendorf, Switzerland), followed by pressing in cheese cloth for 24 h at room temperature. Pomace was then freeze dried and blended in a Model 7011C commercial blender (Conair, Stamford, CT, USA) with 40 second pulses until it could pass a sieve size of 1.18 mm. Blended pomace was suspended (13.8%, w/v) in ethanol (95%, v/v), shaken at 150 rpm for 1 h and filtered on Miracloth rayon-polyester cloth (Millipore Sigma, Burlington, MA, USA). The residues (46%, w/v) were washed three times with 85% ethanol, 50% chloroform, 50% methanol and acetone, then dried overnight at room temperature yielding the alcohol insoluble solids (AIS).

5.3.3. Sequential extraction of polysaccharides

A four-step sequential approach for the extraction of polysaccharides from 8000 mg of dry AIS was implemented adapting the procedure described by Hilz et al. (2005). Briefly, AIS (2.66% w/v) were suspended twice in 0.05 M, pH of 5.2 sodium acetate buffer with the addition of 0.05 M ethylenediaminetetraacetic acid (EDTA) and 0.05 M sodium oxalate at 70 °C for 15 min. The suspension was then centrifuged at 8000 ×G for 30 min and filtered on fritted glass funnel of medium pore size, recovering the supernatant (chelating agents extract, CH). The precipitate was washed once with distilled water at room temperature (3% w/v) to remove excess chelating agents, then suspended twice (2.66% w/v) in 0.05 M sodium hydroxide at 0 °C for 60 min.

The suspension was then brought to pH of 5.2 with acetic acid, centrifuged and filtered, obtaining the diluted alkali extract (DA). The extracts were concentrated by ultrafiltration on a Prep/Scale Spiral Wound module (MilliporeSigma). The retentates were dialyzed with a cut-off of 5000-8000 Da: DA was dialyzed in distilled water for 48 h; CH was dialyzed for 24 h in 0.1 M sodium chloride and 24 h in distilled water. Dialyzed extracts were then freeze dried.

5.3.4. Fractionation of polysaccharides by anion exchange chromatography

The polysaccharide extracts were fractioned by anion exchange chromatography with an ÄKTApurifier UPC 10, on Source 15Q column (GE Healthcare Life Sciences). Elution was conducted at constant flow rate 1.5 ml/min with one column volume of 0.005 M sodium acetate buffer at pH of 5, followed by a linear gradient for 7 column volumes up to 1.5 M. The final molarity was kept for one column volume before re-equilibrating the system with the initial molarity. Fractions containing high concentrations of uronic and/or neutral sugars were pooled, dialyzed in water and freeze dried.

5.3.5. Purification of polysaccharides by gel filtration chromatography

Selected anion exchange fractions were further purified on Sephacryl S-1000 SF resin (GE healthcare), a size exclusion chromatography column. The elution was carried out with sodium phosphate buffer (0.05 M, pH 7) containing 0.15 M sodium chloride at a flow rate of 0.5 ml/min. Fractions containing polysaccharides were pooled, dialyzed against distilled water with cut-off 5-8 kDa and freeze dried.

5.3.6. Enzymatic fragmentation of selected polysaccharides

Purified PPS (0.04 mg/ml) obtained upon gel filtration were suspended in sodium acetate buffer (0.05 M, pH 4.5). Enzymatic reactions were conducted at 40 °C, under 150 rpm shaking in an Excella E24 orbital shaker (New Brunswick Scientific, Edison, NJ, USA). The enzymatic treatment was first carried with pectin lyase (EC 4.2.2.10) from *Aspergillus niger* (1.4%, w/w enzyme to polysaccharide ratio). After 42 h of reaction, *endo*-polygalacturonase (EC 3.2.1.15) from *Aspergillus aculeatus* (1.2%, w/w) was added to the reaction mixtures, and the reactions were carried out for 72 h. In the last stage of enzymatic treatments, either *endo*-α-(1,5)-arabinanase (EC 3.2.1.99) from *A. niger* (6.25%, w/w), *endo*-β-(1,4)-galactanase (EC 3.2.1.89) from *A. niger* (0.13%, w/w) or both together were added an allowed to react for 72 h. Reactions were halted by enzyme inactivation (100 °C for 5 min). Enzymatic reactions were monitored at various times by determining the increase in sugar reducing ends using 3,5-Dinitrosalicylic acid (DNS) test.

Briefly, 0.1 ml of aliquots of enzymatic reactions were added to 1.5 ml of DNS 1% w/v in 1.6% w/v sodium hydroxide and 0.9 ml of water. After heating at 100 °C for 5 min, 0.5 ml of potassium sodium tatrate 50% w/v were added. Absorbance at 540 nm was read once the mixture reached room temperature. All reactions were performed in duplicate. Upon the sequential enzymatic fragmentation, the solubilized fragments were separated from polysaccharides by adding an aliquot of reaction mixture (5% v/v) to ethanol. The mixtures were allowed to settle for 5 h at -20 °C, then centrifuged for 25 min at 6708 ×G to recover the soluble fragments in the supernatant.

5.3.7. Sugar content and monosaccharide profile

Uronic acid content was measured by sulphamate/m-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973). The phenol–sulphuric acid colorimetric assay was used for the determination of neutral sugar content (DuBois et al., 1956). In order to determine the monosaccharide profile, fractions were first hydrolyzed using a two-step procedure as previously described (Khodaei & Karboune, 2013). The samples were suspended and incubated at 60 °C for 24 h in HCl/methanol mixture (1:4, v/v) at a ratio of 0.6% (w/v) and thereafter boiled for 1 h in trifluoroacetic acid solution at a ratio of 1:8 (v/v). Hydrolyzed samples were analyzed with high performance anionic exchange chromatography, equipped with pulsed amperometric detector (HPAEC-PAD, Dionex), and a CarboPac PA20 column (3 × 150 mm). Isocratic elution was performed with 5 mM NaOH (0.5 ml/min). L-Rha, L-Ara, D-Gal, D-Glc, D-Xyl and D-Man were used as standards.

5.3.8. Molecular weight distribution analysis

A high-performance size-exclusion chromatography (HPSEC) system (Model 1525 binary HPLC pump, equipped with a Model 2414 refractive index detector, Waters Co., Milford, MA, USA) was used to estimate the molecular weight distribution in the extracts and their fractions and quantify the yield of polysaccharide extraction. Columns TSK G5000 PWXL and TSK G3000 PWXL (Tosoh Co., Yamaguchi, Japan) were used in series with isocratic flow rate of 0.4 ml/min of 0.1 M sodium chloride. Detector was operated at 30 °C. Dextrans (50 - 670 kDa) and soybean rhamnogalacturonan (0.3 - 5 mg/ml) were used as standards for calibration.

5.3.9. Preparation of methylated PMP-monosaccharides

Ethanol solutions containing the polysaccharide fragments (0.2 ml) were evaporated at 50 °C until dry in screw cap tubes.

The solids were dissolved in 0.5 ml of dimethyl sulfoxide, and 20 mg of freshly powdered sodium hydroxide were added. Iodomethane (0.1 ml) was then added, and the mixtures were gently shaken at room temperature for 10 min. Permethylated samples were recovered by extraction with dichloromethane (1:1. v/v). The organic phase was washed three times with water and dried. Hydrolysis to monosaccharides was performed by incubation with 0.5 ml trifluoroacetic acid at 100 °C for 1 h. Methylated monosaccharides were then dissolved in 0.05 ml water and derivatized by addition of 0.2 ml ammonia solution (28.0–30.0%) and 0.2 ml of 0.2 M 1-phenyl-3-methyl-5-pyrazolone (PMP) in methanol. The solution was heated at 70 °C for 30 minutes and dried under nitrogen.

5.3.10. Sugar linkage analysis by liquid chromatography-mass spectrometry

The obtained methylated PMP-monosaccharides were analyzed by liquid chromatography mass spectrometry using an Agilent 1290 Infinity II LC system coupled to the 6545 Q-TOF -MS (Agilent Technologies, Santa Clara, USA). The LC separation was conducted on a Poreshell120 EC-C18 analytical column (Agilent Technologies). The mobile phase A was HPLC water with 5 mM ammonium acetate and the mobile phase B was acetonitrile/methanol mixture (50:50 v/v) with 5mM ammonium acetate. HPLC parameters were as follows: injection volume was 1 µL, the flow rate was 0.4 mL/min and the column temperature was set to 35°C. The mobile phase profile used for the run in positive ion mode was 10% B (0 to 1.0 min), linear increase to 99% B (1.0 to 8.0 min), hold at 99% B (8.0-13.0 min), decrease to 10% B (13.0 to 13.01 min) and finally 10% B (13.01 to 16 min). The mass spectrometer was equipped with a Dual AJS ESI ion source operating in positive ionization modes. MS conditions were as follows: for ESI+, the drying gas temperature was 275 °C, drying gas flow rate was 10 L/min, sheath gas temperature was 300°C, sheath gas flow rate was 12 L/min, the pressure on the nebulizer was 45 psi, the capillary voltage was 3500 V, the fragmentor voltage was 150 V, the skimmer voltage was 50 V and the nozzle voltage was 1000 V. Full scan MS data were recorded between mass-to-charge ratios (*m/z*) 100 and 1100 at a scan rate of 2 spectra/s, and were collected at both centroid and profile mode. All Ions MS/MS data was collected between *m/z* 100 and 1100 at a scan rate of 2 spectra/s for four different collision energies (0, 10, 20, and 40 V). Target MS/MS data were collected using collision energy at 30 V. Reference ions (*m/z* at 121.0508 and 922.0098 for ESI+) were used for automatic mass recalibration of each acquired spectrum. Data treatment was conducted using Quantitative Analysis B.07.01 from Agilent MassHunter Workstation Software. Three standard oligo- and polysaccharides, raffinose, pectic galactan and pectic arabinan, were used to confirm the retention time and linkage information.

5.4. Results and discussion

5.4.1. Fractionation and purification of cell wall pectic polysaccharides fractions

The cell wall polysaccharides were isolated from cranberry pomace following a sequential extraction method that involves hot buffer, CH, DA, and strong alkali extraction (Chapter IV. Extraction and Characterization of Cell Wall Polysaccharides from Cranberry (*Vaccinium macrocarpon* var. Stevens) Pomace. CH and DA extracts were found to contain abundant uronic acids, indicating the presence of PPS. The fractionation of CH and DA extracts by anionic exchange chromatography confirmed these findings, as it led to the identification of multiple uronic acid-based polysaccharides associated to variable levels of neutral sugars (Table 5.1). In the CH extract, three main fractions (CH1, CH2, CH3) were eluted at different ionic strengths. These fractions were characterized by a high content of uronic acid (> 90%, mol), indicating the presence of abundant homogalacturonan structures. On the other hand, among the three uronic acid peaks identified in DA extract, the two eluted at lower ionic strength (DA1 and DA2) were accompanied by significantly higher amounts of neutral sugars, while DA3 was comparable with the CH fractions. The fractions rich in neutral sugars represent populations of PPS that possess more neutral branches, such as the arabinan, galactans and arabinogalactans of rhamnogalacturonan type I. These marked differences in the fractionation of cranberry CH and DA extracts set them apart from those obtained from soybean (Huisman et al., 1998), that presented nearly identical fractionation profile. The CH extract of grapes showed uronic acid-rich fractions at 0.4 and 0.7 M ionic strength, the latter accompanied by significantly higher proportion of neutral sugars than what was observed in cranberry (Saulnier & Thibault, 1987). Upon anion exchange chromatography, the CH extract of the fruit of *Dacryodes edulis* displayed a small neutral sugar peak at initial ionic strength 0.05 M, and a single major peak containing both uronic and neutral sugars at 0.2 M (Missang et al., 2001). DA extracts from *Solanum nigrum* berries displayed two peaks: the major one eluted with distilled water, the second at 0.1 M ionic strength (Ding et al., 2012).

The three uronic acid-containing peaks detected in CH and DA anionic chromatography profiles were then pooled and further analyzed by gel filtration chromatography (Figure 5.1). While all fractions showed polydispersity, the distribution of molecular weights differed.

Table 5.1 Total sugar content and uronic acid percentage of the fractions obtained by anion exchange chromatography of the extracts.

Fraction	Ionic strength (M)	Total sugars (% mol/mol in whole extract)	Uronic acid (% mol)
CH1	0.31	33.5 ± 2.9	99.0 ± 0.9 [a]
CH2	0.62	31.5 ± 3.0	91.6 ± 5.2 [a]
CH3	0.97	27.5 ± 5.8	96.1 ± 4.3 [a]
DA1	0.52	24.2 ± 3.7	52.9 ± 9.9 [b]
DA2	0.81	28.3 ± 4.4	54.6 ± 11.8 [b]
DA3	1.08	15.4 ± 2.2	85.6 ± 10.9 [a]

Values represented as average ± standard deviation.

CH1-3: Fractions from chelating agents extract; DA1-3: fractions from diluted alkali extract.

a-b: Means with different letters are significantly different at $P \leq 0.05$.

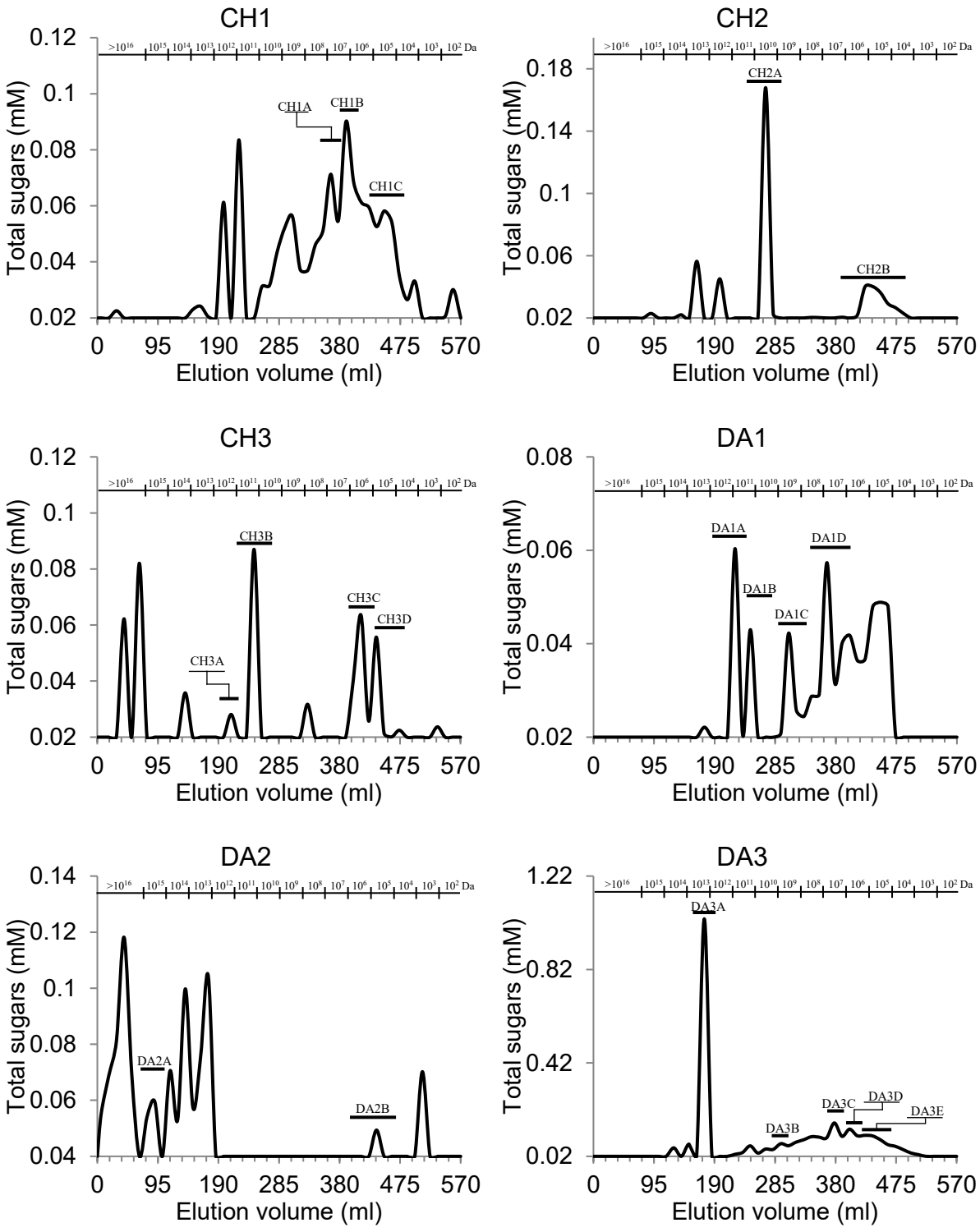

Figure 5.1 Size exclusion chromatography of selected polysaccharide fractions isolated upon anionic exchange fractionation of chelating agents (CH) and diluted alkali (DA) extracts.

CH1 and DA1 fractions contained high percentages of relatively smaller molecular weight compounds (distributed around 10^7 Da), while in CH3 and DA2 fractions, higher molecular weights (10^{11} Da and higher) were abundant, and CH2 and DA3 fractions contained one major polysaccharide peak (around 10^{10} and 10^{13} Da, respectively), with some much smaller peaks at higher and lower (10^6 Da) molecular weights. These results suggest that the uronic acid-containing PS eluted at low ionic strength in the anionic exchange chromatography tend to be shorter than those eluted at high ionic strength. The molecular weight of PPS obtained by diluted acids extraction from hawthorn fruit (Guo et al., 2019) and potato pulp (J.-S. Yang et al., 2019), and those found in water extracts from lingonberry (Ross et al., 2015) were considerably lower (~$1.1 \cdot 10^5$ Da, in the range of $2.0 \cdot 10^4$ Da to $9.5 \cdot 10^5$ Da, and ~$1.2 \cdot 10^5$ Da respectively).

5.4.2. Monosaccharide profile of purified pectic polysaccharides

Selected uronic acid-rich fractions from the gel filtration were pooled, dialyzed, freeze dried and characterized by monosaccharide profile (Table 5.2). All fractions contained more than 70.7% of uronic acids, indicating a prevalence of homogalacturonan regions. CH1B, DA2A and DA3A contained the lowest proportions (<50%) of neutral monosaccharides associated to rhamnogalacturonan type I (RGI) (rhamnose, arabinose and galactose) indicating that in these fractions, RGI structures are not as prevalent. Higher proportions of RGI were detected in CH1C, CH2A and DA3D. The ratio of arabinose and galactose to rhamnose was highest in CH2B (6.42) and DA2A (5.5), suggesting the presence of more or longer branches in these fractions. CH3B, CH3D and DA3D fractions, characterized by the lowest percentages of uronic acids, showed the highest glucose and xylose/mannose contents, indicating the presence of hemicellulose fragments coeluting with these PPS. The existence of covalent bonds between pectic and non-pectic polysaccharide in plant cell wall has been suggested by several studies (Broxterman & Schols, 2018; Popper & Fry, 2008; Zykwinska et al., 2005).

5.4.3. Fragmentation with homogalacturonan-degrading enzymes

The PPS were subjected to sequential enzymatic treatments with pectin lyase and *endo*-polygalacturonase, which have higher substrate specificities towards homogalacturonan backbone. Indeed, pectin lyase hydrolyses the glycosidic bonds between β-(1,4) bound galacturonic acid methyl ester residues of homogalacturonan, while *endo*-polygalacturonase hydrolyzes the same glycosidic bonds between non-methylated residues.

Table 5.2 Molecular weight, yield (determined by gel filtration), monosaccharide composition and pectic polysaccharide-associated neutral sugars (calculated as rhamnose + arabinose + galactose/total neutral sugars) content of selected sub-fractions.

Gel filtration fraction	Yield %	Molecular weight (Da)	Monosaccharide profile (%mol/mol)						Pectic PS neutral sugars (%)
			Rhamnose	Arabinose	Galactose	Glucose	Mannose + Xylose	Uronic acids	
CH1A	15%	$6.54 \cdot 10^{7}$	1.8 ± 0.7 ^{ab}	1.2 ± 0.1 ^{bc}	5.0 ± 1.4 ^{bcd}	2.3 ± 0.0 ^{bc}	1.5 ± 0.1 ^{a}	88.1 ± 6.0 ^{ab}	67.4
CH1B	15%	$1.39 \cdot 10^{7}$	1.9 ± 0.1 ^{ab}	0.9 ± 0.5 ^{bc}	0.2 ± 0.1 ^{d}	1.0 ± 0.6 ^{c}	2.7 ± 0.5 ^{a}	93.3 ± 4.4 ^{a}	44.5
CH1C	17%	$2.92 \cdot 10^{5}$	4.9 ± 2.9 ^{ab}	2.9 ± 1.4 ^{abc}	10.8 ± 0.4 ^{a}	5.9 ± 0.7 ^{abc}	1.3 ± 0.2 ^{a}	74.1 ± 0.7 ^{ab}	72.1
CH2A	27%	$3.18 \cdot 10^{10}$	3.3 ± 0.2 ^{ab}	3.9 ± 2.4 ^{ab}	11.6 ± 0.1 ^{a}	4.4 ± 2.8 ^{abc}	1.6 ± 1.2 ^{a}	75.2 ± 11.8^{ab}	75.8
CH2B	29%	$6.32 \cdot 10^{5}$	0.7 ± 0.4 ^{b}	0.8 ± 0.1 ^{bc}	3.7 ± 0.1 ^{bcd}	2.5 ± 1.5 ^{bc}	1.0 ± 0.1 ^{a}	91.3 ± 2.7 ^{ab}	60.2
CH3A	7%	$1.52 \cdot 10^{12}$	1.3 ± 0.2 ^{b}	1.5 ± 0.2 ^{bc}	2.9 ± 1.9 ^{cd}	2.9 ± 1.9 ^{bc}	1.4 ± 0.2 ^{a}	90.0 ± 3.8 ^{ab}	56.4
CH3B	14%	$1.49 \cdot 10^{11}$	6.3 ± 3.5 ^{ab}	1.7 ± 0.3 ^{bc}	5.8 ± 0.0 ^{abcd}	6.3 ± 1.1 ^{abc}	3.1 ± 2.1 ^{a}	76.8 ± 10.7 ^{ab}	59.5
CH3C	15%	$2.97 \cdot 10^{6}$	2.2 ± 0.4 ^{ab}	1.1 ± 0.5 ^{bc}	6.8 ± 1.1 ^{abc}	4.7 ± 2.4 ^{abc}	1.5 ± 0.0 ^{a}	83.6 ± 6.2 ^{ab}	62.1
CH3D	14%	$6.32 \cdot 10^{5}$	5.2 ± 3.4 ^{ab}	2.3 ± 0.9 ^{abc}	8.9 ± 0.4 ^{ab}	9.6 ± 1.1 ^{a}	3.3 ± 1.8 ^{a}	70.7 ± 11.5 ^{b}	56.2
DA2A	12%	$3.47 \cdot 10^{15}$	0.6 ± 0.3 ^{b}	0.4 ± 0.2 ^{c}	2.9 ± 1.4 ^{bcd}	5.8 ± 1.1 ^{abc}	1.2 ± 0.7 ^{a}	89.1 ± 8.6 ^{ab}	35.6
DA3A	32%	$1.55 \cdot 10^{13}$	2.0 ± 0.3 ^{ab}	0.9 ± 0.1 ^{bc}	2.2 ± 0.4 ^{cd}	7.7 ± 0.3 ^{ab}	1.1 ± 0.0 ^{a}	86.0 ± 8.6 ^{ab}	36.6
DA3C	11%	$3.02 \cdot 10^{7}$	3.2 ± 2.0 ^{ab}	0.8 ± 0.1 ^{bc}	4.5 ± 1.9 ^{bcd}	1.8 ± 0.2 ^{c}	1.6 ± 0.5 ^{a}	88.1 ± 8.2 ^{ab}	71.1
DA3D	7%	$6.43 \cdot 10^{6}$	9.6 ± 5.9 ^{a}	5.0 ± 1.5 ^{a}	8.4 ± 5.3 ^{abc}	2.6 ± 0.1 ^{bc}	2.7 ± 1.3 ^{a}	71.7 ± 10.2 ^{ab}	81.3
DA3E	11%	$6.32 \cdot 10^{5}$	5.0 ± 3.2 ^{ab}	1.4 ± 0.2 ^{bc}	6.9 ± 1.1 ^{abc}	4.2 ± 2.3 ^{abc}	1.3 ± 0.6 ^{a}	81.2 ± 7.5 ^{ab}	70.7

All values are expressed as mean ± SD.

$^{a-d}$: Sample means with different superscript letters in the same column are significantly different ($P \leq 0.05$).

As both pectin lyase and *endo*-polygalacturonase act synergistically to hydrolyse homogalacturonan to alcohol soluble fragments, the release of uronic acid residues (Figure 5.2) can be correlated to the homogalacturonan proportion in the PPS. In fractions CH3A and DA3E, that represent PPS eluted at high ionic strength, uronic acids appeared to be almost entirely part of homogalacturonan, as an average of 88.2% and 95.7% of their total amount appeared in the supernatant at the end of the two enzymatic treatments. On the other hand, fractions that were eluted at lower ionic strength, such as CH1A, CH1B, CH1C and DA2A appeared to possess larger amounts of other uronic acid-containing structures (possibly rhamnogalacturonan and xylogalacturonan backbones). In comparison sugar beet pectin, hydrolyzed by pectin lyase, released 78% of its total content of uronic acid (Holck et al., 2011).

Comparing the amounts of uronic acids released by each enzyme provides information on the abundance of methylated galacturonic acid residues in homogalacturonan. Since all fraction except CH3C released detectable amounts of uronic acids when treated with pectin lyase, it could be concluded that methylation is abundant, with some differences between fractions. As no increase of uronic acid could be observed with *endo*-polygalacturonase treatment of CH1A, CH2B, CH3B, DA2A and DA3A, these fractions appeared to contain high amounts of methylated galacturonic acid in their homogalacturonan, with the non-methylated residues being scattered across the polymer, and thus rendered alcohol-soluble as short oligomers with methylated residues at their extremities. Interestingly, CH3B and DA3A were eluted at high ionic strengths, and as such were expected to contain high amounts of acid residues. The content of non-esterified galacturonic acid that could not be released upon *endo*-polygalacturonase treatment, as it is part of other non-homogalacturonan structures, may account for this discrepancy. On the other hand, in samples such as CH3C, DA3E, CH2A and CH3A, a large portion of the total uronic acid got released by polygalacturonase, indication of the presence of clusters of non-methylated residues in their homogalacturonan chains, since the action of pectin lyase is unable to fragment all the chain into alcohol-soluble oligomers. The proportion of uronic acids liberated by the two enzymes can hence be used as an estimate of the proportion of clusters of non-methylated galacturonic acid residues. As such the fractions DA3E, CH2A and CH1C are expected to be richer in non-methylated homogalacturonan than CH3D, DA3D and DA3C. Onion PPS were found to liberate 62.5% of the total uronic acids, when treated with pectin lyase, while only 18% with *endo*-polygalacturonase (Ishii, 1982).

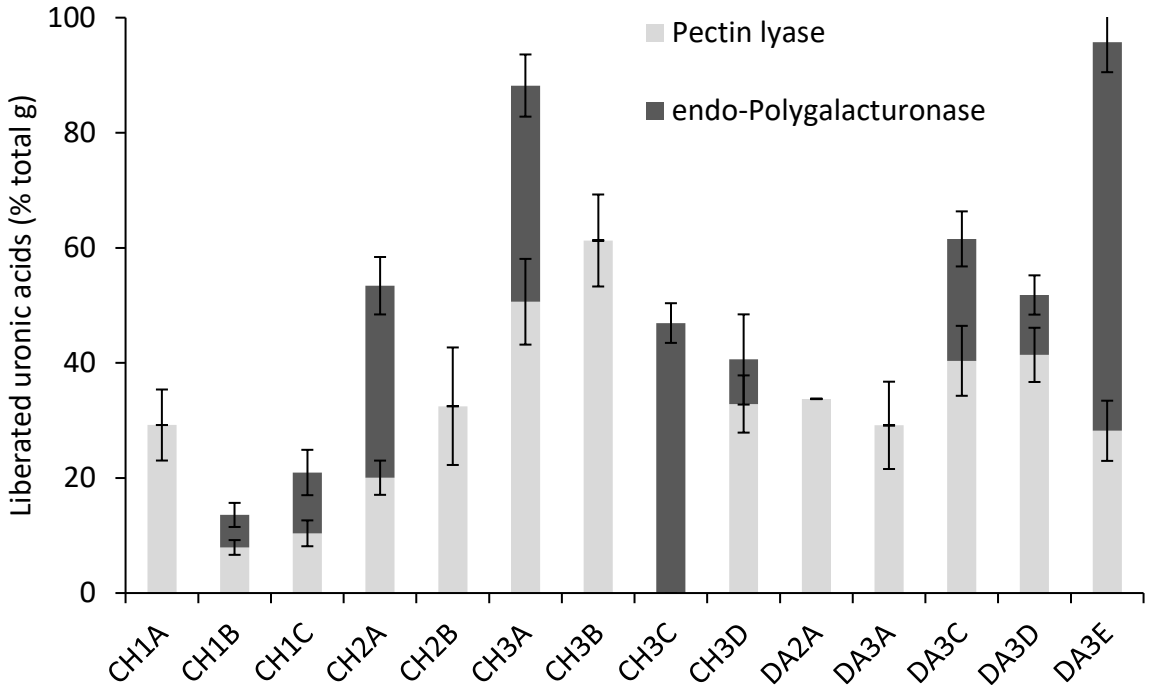

Figure 5.2 Percentage of the fractions' total uronic acids liberated by pectin lyase and *endo*-polygalacturonase sequential treatments.

5.4.4. Fragmentation with debranching glycosyl-hydrolase enzymes

To identify and fragment the RGI neutral sugar branches the fractions, after pectin lyase and *endo*-polygalacturonase treatments, were further subjected to enzymatic treatments with *endo*-α-1,5-arabinanase and *endo*-β-1,4-galactanase either individually or in combination. These two enzymes were selected as a majority of the known most common neutral branches of RGI can be hydrolyzed by their activity (Figure 5.3) (Bonnin et al., 2014). In fact, *endo*-α-1,5-arabinanase has high specificity towards the hydrolysis of 1,5-bound α-L-arabinofuranose residues, suited for fragmenting the backbone and longer branches of pectic arabinan, as well as the branches of type I arabinogalactan. On the other hand, *endo*-β-1,4-galactanase displays high specificity towards the hydrolysis of 1,4-bound β-D-galactopyranose residues, and as such can hydrolyze the backbones of type I arabinogalactan and pectic galactan. The backbone and branches of type II arabinogalactan, which is occasionally found as sidechain of RGI (Leivas et al., 2016), cannot be hydrolyzed by these enzymes as it is a galactose polymer with β-1,3 and β-1,6 linkage.

The fragments generated by the debranching step were hydrolyzed and analyzed by HPAEC-PAD to determine the amount of released RGI-associated monosaccharides (arabinose, galactose and rhamnose) (Table 5.3). *Endo*-α-1,5-arabinanase displayed a generally low ability to release arabinose from most of the fractions, as little arabinose was detectable after this enzymatic treatment. The maximum arabinose percentage was released from DA3E (24.7% of the highest Ara release, which was detected in DA3A after *endo*-β-1,4-galactanase treatment), followed by DA3D and DA3A. This suggests that, in most fractions, arabinose is not α-1,5-linked into arabinan, but is rather found in other types of neutral branch. In fact, comparison with the amount of arabinose liberated by *endo*-β-1,4-galactanase shows that the latter was higher for most of the fractions, except CH1C, DA2A and DA3E, with maximum in DA3A and CH3A. As more arabinose got liberated upon hydrolysis of the glycosidic bond of β-1,4-galactan, type I arabinogalactan that possesses arabinose decorations (Bonnin et al., 2014) may be abundant in the cranberry PPS. Indeed, a type I arabinogalactan structure is commonly found in the cell wall of other fruits, such as orange (Prabasari et al., 2011) could be inferred to be present in larger amounts than arabinan. In extracts from rabbiteye blueberry, a fruit of the same genus as cranberry, it was similarly found that the most abundant type of neutral branch is type I arabinogalactan (Deng et al., 2013).

Endo-β-1,4-galactanase hydrolysis was found to be more effective than *endo*-α-1,5-arabinanase, as it released galactose in detectable amounts from all fractions except CH3D, the maximum being in CH2B.

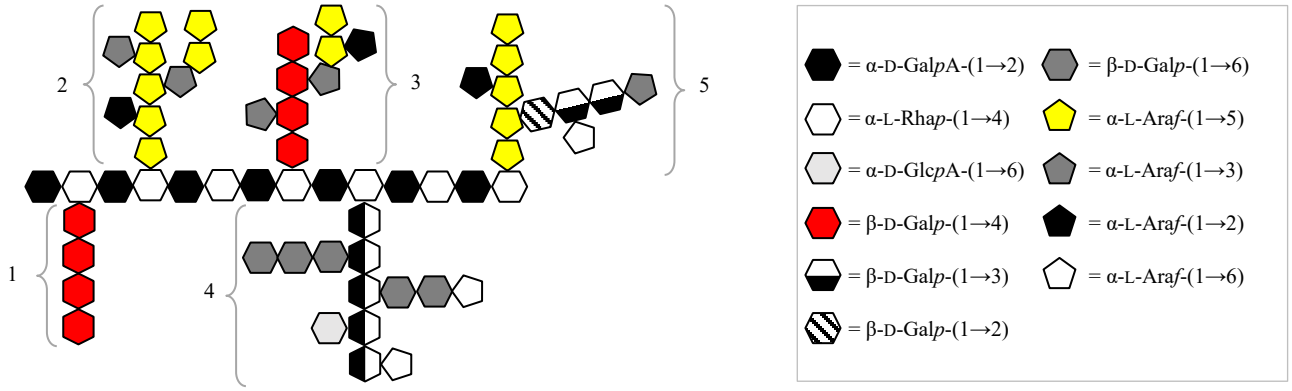

Figure 5.3 Schematic structure of rhamnogalacturonan type I backbone and major branches. 1: galactan; 2: arabinan; 3: type I arabinogalactan; 4: type II arabinogalactan; 5: galactoarabinan. Based on Bonnin, Garnier, & Ralet (2014), Mohnen (2008), Yu et al. (2015)**.**

Table 5.3 Relative recovery of arabinose and galactose in the supernatants after debranching step (% mol in supernatant/ total mol of neutral sugars in fraction).

	endo-α-1,5-arabinanase		endo-β-1,4-galactanase		endo-α-1,5-arabinanase + endo-β-1,4-galactanase	
	Arabinose	Galactose	Arabinose	Galactose	Arabinose	Galactose
CH1A	n.d.	n.d.	12.6 ± 1.4 ^{cd}	20.5 ± 5.0 ^{cd}	21.6 ± 4.3 ^{ab}	24.7 ± 4.6 ^{b}
CH1B	n.d.	87.7 ± 22.9 ^{a}	19.5 ± 3.0 ^{c}	69.1 ± 11.8 ^{b}	n.d.	n.d.
CH1C	0.1 ± 0.0 ^{c}	0.3 ± 0.1 ^{d}	n.d.	0.3 ± 0.1 ^{f}	0.1 ± 0.0 ^{e}	0.2 ± 0.1 ^{f}
CH2A	n.d.	n.d.	1.8 ± 0.4 ^{e}	1.6 ± 0.4 ^{f}	0.9 ± 0.3 ^{d}	2.4 ± 0.8 ^{d}
CH2B	n.d.	n.d.	61.6 ± 5.6 ^{b}	100.0 ± 23.3 ^{a}	0.4 ± 0.1 ^{d}	0.3 ± 0.1 ^{f}
CH3A	0.6 ± 0.0 ^{c}	1.0 ± 0.2 ^{e}	89.6 ± 20.4 ^{a}	14.9 ± 3.2 ^{d}	1.2 ± 0.1 ^{d}	3.5 ± 0.9 ^{d}
CH3B	0.8 ± 0.1 ^{c}	4.7 ± 1.2 ^{d}	1.3 ± 0.3 ^{e}	2.7 ± 0.5 ^{f}	0.6 ± 0.1 ^{d}	1.5 ± 0.3 ^{e}
CH3C	0.9 ± 0.1 ^{c}	0.8 ± 0.1 ^{e}	1.8 ± 0.4 ^{e}	6.2 ± 1.1 ^{e}	0.4 ± 0.1 ^{d}	1.5 ± 0.4 ^{e}
CH3D	n.d.	n.d.	0.3 ± 0.0 ^{f}	n.d.	0.8 ± 0.1 ^{d}	3.2 ± 0.6 ^{d}
DA2A	0.5 ± 0.1 ^{c}	2.5 ± 0.5 ^{d}	n.d.	1.2 ± 0.3 ^{f}	0.5 ± 0.1 ^{d}	0.5 ± 0.2 ^{f}
DA3A	6.3 ± 1.0 ^{b}	6.6 ± 1.7 ^{cd}	100.0 ± 4.1 ^{a}	35.3 ± 7.1 ^{c}	15.7 ± 2.4 ^{bc}	42.5 ± 10.2 ^{a}
DA3C	n.d.	n.d.	11.6 ± 1.7 ^{d}	38.4 ± 6.7 ^{c}	8.5 ± 1.3 ^{c}	17.9 ± 4.2 ^{bc}
DA3D	11.6 ± 0.9 ^{b}	10.8 ± 2.0 ^{c}	9.2 ± 1.4 ^{d}	9.7 ± 1.7 ^{de}	34.0 ± 6.9 ^{a}	13.7 ± 2.4 ^{c}
DA3E	24.7 ± 5.5 ^{a}	32.5 ± 6.5 ^{b}	22.2 ± 4.6 ^{c}	47.4 ± 12.8 ^{bc}	11.6 ± 1.0 ^{c}	4.5 ± 1.1 ^{d}

All values are expressed as mean ± SD.

n.d.: Not detected.

Ara:Gal: Ratio between arabinose and galactose moles in supernatant.

$^{a-f}$: Sample means with different superscript letters in the same column are significantly different ($P \leq 0.05$).

A portion of galactose may also be included in structures that are resistant to the two enzymes, such as monomeric decorations on RGI backbone as well as type II arabinogalactan. As type II arabinogalactan is often found to possess branches containing 1,6-linked arabinose (Ridley et al., 2001), some of the undetected arabinose may be also part of this structure. The presence of type II arabinogalactan in cranberry polysaccharide extracts has been discussed in previous study (Chapter IV. Extraction and Characterization of Cell Wall Polysaccharides from Cranberry (*Vaccinium macrocarpon* var. Stevens) Pomace).

The results (Table 5.3) also show that detectable amounts of galactose were found after treatment with *endo*-α-1,5-arabinanase; structures with arabinan backbone and galactose-containing branches may be present, such as galactoarabinan (Yapo, 2011). While less common in plants than arabinan and arabinogalactan, galactoarabinan have been proposed as RGI branches in sugar beet, where small amounts of galactose could be found in the fragments generated by arabinanase (Sakamoto & Sakai, 1995), as well as in potato (Øbro et al., 2004), in which 1,4-linked galactan was released by arabinanase, and duckweed (Yu et al., 2015), in which galactan is 1,3-linked.

The simultaneous treatment with both debranching enzymes resulted in an increased abundance of arabinose and galactose in the fragments of the CH1A, CH2A, CH3D and DA3D fractions. This effect can be used as an indication of the presence of structures, such as type I arabinan, in which both 1,5-linked arabinan and 1,4-linked galactan coexist, and the hydrolysis of the arabinan branches allows better access of galactanase to the backbone. It should be noted that, aside from CH1A, the samples in which this effect was observed were among those with the highest proportion of arabinose and galactose (Table 5.2). It is therefore possible that in these samples the neutral sidechains are abundant enough to allow detection of the synergistic effect, while for the others the concentration is too low.

5.4.5. Glycoside linkage analysis of polysaccharide fragments recovered upon debranching

The polysaccharide hydrolysates generated upon debranching by glycosyl hydrolase enzymes were analyzed by LC-MS after conversion to methylated and PMP-labeled monosaccharides. Table 5.4 shows the relative molar proportions of the linkages present in the fragments obtained upon enzymatic debranching of CH1B, DA4A and DA4E. Although the presence of glycosidic residues involved in multiple glycosidic bonds was likely overestimated, due to the incomplete methylation of the residues, the characteristics of the polysaccharide fragments were discussed.

Table 5.4 Glycosidic residue linkages (%mol) of the fragments obtained upon enzymatic treatment of polysaccharides. e-Ara: treatment with pectin lyase, polygalacturonase and *endo*-arabinanase; e-Gal: treatment with pectin lyase, polygalacturonase and *endo*-galactanase. both: treatment with pectin lyase, polygalacturonase and *endo*-arabinanase + *endo*-galactanase.

		CH1B			DA3A			DA3E		
m/z	Linkage pattern	e-Ara	e-Gal	both	e-Ara	e-Gal	both	e-Ara	e-Gal	both
523.3	T-Ara	0.8	0.0	1.1	1.0	7.5	2.2	0.8	2.4	0.3
509.2	1,n-Ara	5.6	22.2	4.0	2.9	19.0	8.2	0.0	0.0	2.8
495.2	1,3,n-Ara	6.4	3.6	4.5	3.4	0.0	8.4	17.9	20.9	6.0
481.2	1,2,3,5-Ara	19.5	50.4	55.7	54.0	7.6	26.6	34.6	57.3	55.7
Total Arabinose		**32.3**	**76.2**	**65.4**	**61.2**	**34.1**	**45.4**	**53.2**	**80.6**	**64.8**
567.3	T-Gal	0.9	0.0	0.4	0.8	8.5	1.3	1.6	0.0	0.0
553.3	1,n-Gal	11.0	0.0	9.4	13.9	26.1	9.3	2.3	0.0	10.8
539.3	1,n,n-Gal	19.6	0.0	8.4	8.1	0.0	13.5	6.6	0.0	8.2
525.2	1,3,4,6-Gal	19.0	0.0	6.5	6.3	0.0	7.7	0.0	0.0	7.6
525.2	1,2,4,n-Gal	9.6	9.6	2.7	3.8	0.0	8.7	32.8	5.6	4.4
Total Galactose		**60.0**	**9.6**	**27.3**	**33.0**	**34.7**	**40.5**	**43.4**	**5.6**	**30.9**
523.3	1,2-Rha	0.8	0.0	1.1	1.0	7.5	2.2	0.8	2.4	0.3
509.2	1,n,n-Rha	5.6	8.0	4.3	2.9	19.0	8.2	0.0	0.0	2.8
Total Rhamnose		**6.4**	**8.0**	**5.5**	**3.9**	**26.5**	**10.4**	**0.8**	**2.4**	**3.1**
567.3	T-Glc	0.6	4.8	1.2	0.6	4.7	2.5	2.7	9.7	0.6
495.2	1,3,4-Xyl	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0
567.2	1,n-GalA	0.7	1.4	0.7	0.8	0.0	1.1	0.0	1.7	0.6

In all investigated fractions, arabinose and galactose represented most of the detected residues, coherently with the expected hydrolytic activity of the debranching enzymes. Arabinose and galactose occupied slightly larger percentages of the monosaccharide profile of the fragments generated by *endo*-α-1,5-arabinanase than by *endo*-β-1,4-galactanase, as the latter contained higher amounts of glucose, rhamnose and, in the case of CH1B and DA3E, galacturonic acid. The presence of more glucose in the fragments liberated by *endo*-β-1,4-galactanase suggests that non-pectic structures may be associated with pectic galactan and type I arabinogalactan and released upon the enzymatic hydrolysis of those chains. The ratios of 1,3,n-Ara moles to total arabinose moles in the fragments obtained by *endo*-α-1,5-arabinanase provide an indication of the extent of arabinan branching, which resulted higher in DA3E (ratio 0.35), followed by CH1B (0.20) and finally DA3A (0.05).

Upon treatment with *endo*-β-1,4-galactanase, fractions CH1B and DA3E released a limited amount of galactose, indicating that in these fractions the β-(1,4)-bound galactopyranose of galactan and type I arabinogalactan may not be abundant. On the other hand, since a large portion of these fragments was composed of arabinose, a type I arabinogalactan with short backbone of galactose highly branched with arabinose and arabinans would fit the observations. Branching would in fact hinder the galactanase activity, and the generated fragments would include some of the arabinose-containing branches. Furthermore, simultaneous addition of both debranching enzymes yielded fragments with more abundant arabinose than those from arabinanase and much more abundant galactose than those from galactanase. This effect suggests that the action of arabinanase significantly improves the activity of galactanase, as would be the case in the presence of arabinan branches on type I arabinogalactan. An analogous synergistic effect between these two enzymes was reported in the hydrolysis of potato type I arabinogalactan (van de Vis et al., 1991). The presence of some 1,2,4,n-Gal linkage in these fragments could also reflect the presence of branching points in the arabinogalactan backbone, as they might represent incompletely methylated 1,2,4-Gal residues.

In all the fractions *endo*-α-1,5-arabinanase released a significant amount of galactose residues, with linkages that reflect the presence of linear (1,n-Gal) and branched (1,n,n-Gal) galactose-containing structures. In particular, the proportion of galactose that this enzyme released from CH1B and DA3E was much higher than the one that galactanase did. Overall, these results characterize the neutral branches of CH1B and DA3E as mainly type I arabinogalactan, characterized by β-(1,4) galactan backbones with extended arabinan branches.

Coherently with the analysis by HPSEC, the presence of α-(1,5) arabinan backbones with galactan branches are likely present in these fractions, but further analysis of the oligosaccharides in the fragments will be needed to verify the presence of this hypothesized galactoarabinan. On the other hand, in DA3A fragments, a higher release of galactose residues was obtained upon *endo*-β-1,4-galactanase treatment as compared to CH1B and DA3E fragments. This observation, combined with the lack of detectable galactose residues involved in more than two glycosidic bonds (1,n,n-Gal, 1,2,4,6-Gal and 1,2,4,n-Gal), provides a strong indication that DA3A contains 1,4-bound galactan backbones (pectic galactan), which are characterized by low branching (Srivastava, 2002). The presence of arabinose in this fraction suggests that some type I arabinogalactan structures may also be present, possibly analogous to the ones reported in sugar beet, where some arabinans are not connected to RGI backbone directly, but through a galactose residue or very short galactan chain (Ralet et al., 2009).

5.5. Conclusions

PPS were identified in the extracts obtained by chelating agents and diluted alkali extraction from Stevens variety cranberries pomace. Selected molecular weight populations of these PPS were collected, purified and further analyzed to determine the monosaccharide composition and their structures. Extensively methylated homogalacturonan, type I arabinogalactan, and a smaller amount of linear galactan were identified and discussed, and indications of arabinan structures with galactose-containing branches were found. A deeper characterisation of the structures of extractable cranberry pectic polysaccharides is expected to contribute to the understanding of cranberry cell wall structure and to the development of approaches targeted at the isolation of specific polysaccharides and of the oligosaccharides that can be obtained from their hydrolysis.

5.6. References

Blumenkrantz, N., & Asboe-Hansen, G. (1973). "New method for quantitative determination of uronic acids". Analytical Biochemistry *54*(2): 484–489.

Bonnin, E., Garnier, C., & Ralet, M.-C. (2014). "Pectin-modifying enzymes and pectin-derived materials: applications and impacts.". Applied Microbiology and Biotechnology *98*(2): 519–532.

Broxterman, S. E., & Schols, H. A. (2018). "Interactions between pectin and cellulose in primary plant cell walls". Carbohydrate Polymers *192*: 263–272.

Deng, J., Shi, Z.-J., Li, X.-Z., & Liu, H.-M. (2013). "Soluble polysaccharides isolation and characterization from rabbiteye blueberry (Vaccinium ashei) fruits". BioResources *8*(1): 405–419.

Ding, X., Zhu, F., & Gao, S. (2012). "Purification, antitumour and immunomodulatory activity of water-extractable and alkali-extractable polysaccharides from Solanum nigrum L.". Food Chemistry *131*(2): 677–684.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). "Colorimetric Method for Determination of Sugars and Related Substances". Analytical Chemistry *28*(3): 350–356.

Guo, Q., Du, J., Jiang, Y., Goff, H. D., & Cui, S. W. (2019). "Pectic polysaccharides from hawthorn: Physicochemical and partial structural characterization". Food Hydrocolloids *90*: 146–153.

Hilz, H., Bakx, E. J., Schols, H. A., & Voragen, A. G. J. (2005). "Cell wall polysaccharides in black currants and bilberries—characterisation in berries, juice, and press cake". Carbohydrate Polymers *59*(4): 477–488.

Holck, J., Hjernø, K., Lorentzen, A., Vigsnæs, L. K., Hemmingsen, L., Licht, T. R., … Meyer, A. S. (2011). "Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after in vitro fermentation". Process Biochemistry *46*(5): 1039–1049.

Hotchkiss, A. T., Nuñez, A., Strahan, G. D., Chau, H. K., White, A. K., Marais, J. P. J., … Khoo, C. (2015). "Cranberry Xyloglucan Structure and Inhibition of Escherichia coli Adhesion to Epithelial Cells". Journal of Agricultural and Food Chemistry *63*(23): 5622–5633.

Huisman, M. M. ., Schols, H. ., & Voragen, A. G. . (1998). "Cell wall polysaccharides from soybean (Glycine max.) meal. Isolation and characterisation". Carbohydrate Polymers *37*(1): 87–95.

Ishii, S. (1982). "Enzymatic extraction and linkage analysis of pectic polysaccharides from onion". Phytochemistry *21*(3): 778–780.

Khodaei, N., & Karboune, S. (2013). "Extraction and structural characterisation of rhamnogalacturonan I-type pectic polysaccharides from potato cell wall". Food Chemistry *139*(1–4): 617–623.

Kratchanova, M., Nikolova, M., Pavlova, E., Yanakieva, I., & Kussovski, V. (2010). "Composition and properties of biologically active pectic polysaccharides from leek (Allium porrum).". Journal of the Science of Food and Agriculture *90*(12): 2046–51.

Leivas, C. L., Iacomini, M., & Cordeiro, L. M. C. (2016). "Pectic type II arabinogalactans from starfruit (Averrhoa carambola L.)". Food Chemistry *199*: 252–257.

Li, P., Xia, J., Nie, Z., & Shan, Y. (2016). "Pectic oligosaccharides hydrolyzed from orange peel by fungal multi-enzyme complexes and their prebiotic and antibacterial potentials". LWT - Food Science and Technology *69*: 203–210.

Li, S., Li, T., Zhu, R., Wang, N., Song, Y., Wang, S., & Guo, M. (2013). "Antibacterial Action of Haw Pectic Oligosaccharides". International Journal of Food Properties *16*(3): 706–712.

Manderson, K., Pinart, M., Tuohy, K. M., Grace, W. E., Hotchkiss, A. T., Widmer, W., … Rastall, R. A. (2005). "In Vitro Determination of Prebiotic Properties of Oligosaccharides Derived from an Orange Juice Manufacturing By-Product Stream". Applied and Environmental Microbiology *71*(12): 8383–8389.

Missang, C. E., Renard, C. M. G. C., Baron, A., & Drilleau, J.-F. (2001). "Cell wall polysaccharides of bush butter (Dacryodes edulis (G Don) HJ Lam) fruit pulp and their evolution during ripening". Journal of the Science of Food and Agriculture *81*(8): 773–780.

Mohnen, D. (2008). "Pectin structure and biosynthesis.". Current Opinion in Plant Biology *11*(3): 266–277.

Øbro, J., Harholt, J., Scheller, H. V., & Orfila, C. (2004). "Rhamnogalacturonan I in Solanum tuberosum tubers contains complex arabinogalactan structures.". Phytochemistry *65*(10): 1429–38.

Oechslin, R., Lutz, M. V, & Amadò, R. (2003). "Pectic substances isolated from apple cellulosic residue: structural characterisation of a new type of rhamnogalacturonan I". Carbohydrate Polymers *51*(3): 301–310.

Olano-Martin, E., Gibson, G. R., & Rastall, R. A. (2002). "Comparison of the in vitro bifidogenic properties of pectins and pectic-oligosaccharides". Journal of Applied Microbiology *93*(3): 505–511.

Olano-Martin, E., Mountzouris, K. C., Gibson, G. R., & Rastall, R. A. (2001). "Continuous Production of Pectic Oligosaccharides in an Enzyme Membrane Reactor". Journal of Food Science *66*(7): 966–971.

Oosterveld, A., Beldman, G., Schols, H. A., & Voragen, A. G. J. (1996). "Arabinose and ferulic acid rich pectic polysaccharides extracted from sugar beet pulp". Carbohydrate Research *288*: 143–153.

Peña, M. J., & Carpita, N. C. (2004). "Loss of highly branched arabinans and debranching of rhamnogalacturonan I accompany loss of firm texture and cell separation during prolonged storage of apple.". Plant Physiology *135*(3): 1305–13.

Popper, Z. A., & Fry, S. C. (2008). "Xyloglucan-pectin linkages are formed intra-protoplasmically, contribute to wall-assembly, and remain stable in the cell wall". Planta *227*(4): 781–794.

Prabasari, I., Pettolino, F., Liao, M.-L., & Bacic, A. (2011). "Pectic polysaccharides from mature orange (Citrus sinensis) fruit albedo cell walls: Sequential extraction and chemical characterization". Carbohydrate Polymers *84*(1): 484–494.

Ralet, M.-C., Guillon, F., Renard, C., & Thibault, J.-F. (2009). "Sugar Beet Fiber". In *Fiber Ingredients* (pp. 359–392). CRC Press.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). "Pectins: structure, biosynthesis, and oligogalacturonide-related signaling". Phytochemistry *57*(6): 929–967.

Ross, K. A., Godfrey, D., & Fukumoto, L. (2015). "The chemical composition, antioxidant activity and α-glucosidase inhibitory activity of water-extractable polysaccharide conjugates from northern Manitoba lingonberry". Cogent Food & Agriculture *1*(1): 1109781.

Sakamoto, T., & Sakai, T. (1995). "Analysis of structure of sugar-beet pectin by enzymatic methods". Phytochemistry *39*(4): 821–823.

Saulnier, L., & Thibault, J.-F. (1987). "Extraction and characterization of pectic substances from pulp of grape berries". Carbohydrate Polymers *7*(5): 329–343.

Schols, H. A., Bakx, E. J., Schipper, D., & Voragen, A. G. J. (1995). "A xylogalacturonan subunit present in the modified hairy regions of apple pectin". Carbohydrate Research *279*: 265–279.

Srivastava, L. M. (2002). "Cell Wall, Cell Division, and Cell Growth". In *Plant Growth and Development* (pp. 23–74). Elsevier.

Sun, J., Marais, J. P. J., Khoo, C., LaPlante, K., Vejborg, R. M., Givskov, M., … Rowley, D. C. (2015). "Cranberry (Vaccinium macrocarpon) oligosaccharides decrease biofilm formation by uropathogenic Escherichia coli". Journal of Functional Foods *17*: 235–242.

Theuwissen, E., & Mensink, R. P. (2008). "Water-soluble dietary fibers and cardiovascular disease". Physiology & Behavior *94*(2): 285–292.

Tingirikari, J. M. R. (2019). "In-Vitro Prebiotic Analysis of Microbiota Accessible Pectic Polysaccharides". Current Microbiology *76*(12): 1452–1460.

van de Vis, J. W., Searle-van Leeuwen, M. J. F., Siliha, H. A., Kormelink, F. J. M., & Voragen, A. G. J. (1991). "Purification and characterization of Endo-1,4-β-D-galactanases from Aspergillus niger and Aspergillus aculeatus: Use in combination with arabinanases from Aspergillus niger in enzymic conversion of potato arabinogalactan". Carbohydrate Polymers *16*(2): 167–187.

Vidal, S., Doco, T., Williams, P., Pellerin, P., York, W. S., O'Neill, M. A., … Albersheim, P. (2000). "Structural characterization of the pectic polysaccharide rhamnogalacturonan II: evidence for the backbone location of the aceric acid-containing oligoglycosyl side chain". Carbohydrate Research *326*(4): 277–294.

Wang, D., Yeats, T. H., Uluisik, S., Rose, J. K. C., & Seymour, G. B. (2018). "Fruit Softening: Revisiting the Role of Pectin". Trends in Plant Science *23*(4): 302–310.

Yang, J.-S., Mu, T.-H., & Ma, M.-M. (2019). "Optimization of ultrasound-microwave assisted acid extraction of pectin from potato pulp by response surface methodology and its characterization". Food Chemistry *289*: 351–359.

Yapo, B. M. (2011). "Rhamnogalacturonan-I: A Structurally Puzzling and Functionally Versatile Polysaccharide from Plant Cell Walls and Mucilages". Polymer Reviews *51*(4): 391–413.

Yu, L., Yu, C., Zhu, M., Cao, Y., Yang, H., Zhang, X., … Zhou, G. (2015). "Structural analysis of galactoarabinan from duckweed". Carbohydrate Polymers *117*: 807–812.

Zykwinska, A. W., Ralet, M.-C. J., Garnier, C. D., & Thibault, J.-F. J. (2005). "Evidence for In Vitro Binding of Pectin Side Chains to Cellulose". Plant Physiology *139*(1): 397–407.

CONNECTING STATEMENT 4

Having obtained an in-depth characterization of the composition and structure of the cell wall pectic polysaccharides found in cranberry cell wall extracts (chapters IV and V), the research focus shifted to the assessment of the possibility of enzymatic modification of these molecules, and the impact that modification can have on their properties as food ingredients. The results of these investigations are reported in chapter VI. In light of recent findings on the potential as prebiotics of several pectic polysaccharides (Tingirikari, 2019), the fermentability of the extracts by beneficial gut microorganisms was tested, with comparison to prebiotic inulin. As esterification of oligosaccharides with ferulic acid has been connected to modulation of prebiotic effect (Gong et al., 2019), the possibility to esterify the polysaccharide extracts using immobilized *Humicola insolens* feruloyl esterase, an enzyme that had shown esterification of carbohydrates in low water environments, was tested.

Spadoni Andreani, E., Li, M., Ronholm, J. & Karboune, S. (2020). Biocatalyzed Generation of Feruloylated Polysaccharides from Cranberry and Characterization of their Prebiotic Properties. Food Bioscience (*Submitted*).

CHAPTER VI. BIOCATALYZED GENERATION OF FERULOYLATED POLYSACCHARIDES FROM CRANBERRY AND CHARACTERIZATION OF THEIR PREBIOTIC PROPERTIES

6.1. Abstract

Humicola insolens feruloyl esterase was immobilized on chemically modified epoxy-activated supports of different pore sizes. Native and immobilized feruloyl esterases were investigated for their esterification activity on raffinose in surfactantless microemulsions. Pectic polysaccharides, obtained from cranberry pomace by chelating agent (CH) and diluted alkaline (DA) extractions, were then esterified at the optimized conditions. Using CH polysaccharide extract as a substrate, immobilized feruloyl esterase led to higher feruloylation (22.1 to 26.6%) than the free enzyme (4.2%), and yielded polysaccharides with a higher increase in the total phenolic content. Free and immobilized feruloyl esterases performed similarly for the feruloylation of polysaccharides present in the DA extract, identified of being rich in rhamnogalacturonan I neutral branches. The prebiotic activity score study revealed that cranberry pectic polysaccharides were better fermented than inulin and generated abundant propionic acid. Increased feruloylation of cranberry polysaccharides had a negative impact on the growth of *Lactobacillus brevis* and *Bifidobacterium longum* strains. The total short chain fatty acid concentration showed that the presence of feruloylation affected DA polysaccharides more than CH ones. This revealed the importance of controlling the feruloylation extent in order to compromise between the prebiotic and the antioxidant properties.

6.2. Introduction

Pectic polysaccharides are one of the major constituents of plant cell walls and have several applications as food ingredients due to their thickening, gelling, and emulsifying properties (Turquois et al., 1999; Zhang et al., 2020). These polysaccharides are available in abundance and can be inexpensively isolated from food processing by-products, such as fruit pomaces. For instance, cranberry pomace and potato pulp as by-products of juice and potato processing industries were identified as potential sources of pectic polysaccharides (Khodaei & Karboune, 2013; Chapter IV). Several pectic polysaccharides and their corresponding oligosaccharides were found to possess prebiotic activity, as they can be selectively fermented by beneficial bacteria in the human colon, which promotes the production of short chain fatty acids (SCFA) (Gómez et al., 2014; Tingirikari, 2019). Some of SCFA have been associated with intestinal health-promoting effects, such as butyric acid which has an anti-inflammatory effect (Canani et al., 2011), and propionic acid which mitigates the symptoms of type 2 diabetes (Al-Lahham et al., 2010).

Ferulic acid is another common component of plant cell walls, and cell wall polysaccharides that exhibit various degrees of esterification with ferulic acid occur naturally (Faulds & Williamson, 1999). Examples of abundant feruloylated polysaccharides are arabinoxylans - a major component in the cell walls of cereals - and the pectic polysaccharides found in sugar beet. Feruloylated oligo- and polysaccharides can be obtained by the hydrolysis of these polysaccharides (Ralet et al., 1994; Rudjito et al., 2019). However, because of the complexity and heterogeneity of feruloylated polysaccharides, their isolation from plant sources is challenging due to low yields and inconsistent degrees of feruloylation.

Feruloylated oligo/polysaccharides have distinct complementary antioxidant and prebiotic properties, and are good candidates for the delivery of antioxidant compounds in the lower gastrointestinal tract as non-digestible ingredients (Rudjito et al., 2019; Zhao et al., 2018). In some studies, the presence of feruloyl moieties was reported to contribute not only to the antioxidant activity, but also to the prebiotic property of feruloylated oligo/polysaccharides (Gong et al., 2019; Graf, 1992; Yuan et al., 2005). In contrast, another study found that the presence of feruloyl moieties didn't influence the prebiotic activity of pectic oligosaccharides isolated from sugar beet; indeed, both feruloylated and non-feruloylated pectic oligosaccharides exhibited similar prebiotic activity as fructo-oligosaccharides, a class of well-known prebiotics (Holck et al., 2011).

Enzymatic acylation of carbohydrates with phenolic acids in non-conventional media via carboxylic ester hydrolase-catalyzed reactions is a promising route to produce phenolated glycosides (Couto et al., 2011; Mastihubová et al., 2006). In our previous study, feruloylated xylose, xylobiose, and short-chain xylooligosaccharides were obtained by esterification in water-in-organic solvent microemulsion using immobilized feruloyl esterase from *Humicola insolens* (Tamayo-Cabezas & Karboune, 2019). Xylobiose was a more effective substrate than short-chain xylooligosaccharides (Tamayo-Cabezas & Karboune, 2019). In the present study, the enzymatic feruloylation of polysaccharides by *H. insolens* feruloyl esterase was investigated. The polysaccharides were obtained from cranberry cell wall material and characterized as pectic polysaccharides containing both homogalacturonan and rhamnogalacturonan with neutral branches, where natural feruloylation is mainly localized (Oosterveld et al., 2000). Both free and immobilized feruloyl esterase were used as biocatalysts. The supports used for immobilization were epoxy-activated polyacrylic resins with different pore diameters.

Cranberry pectic polysaccharides and the produced feruloylated polysaccharides were tested for their ability to promote the anaerobic growth *in vitro* of two strains of beneficial gut microorganisms: *Lactobacillus brevis* and *Bifidobacterium longum* in comparison to inulin, a known prebiotic polysaccharide (Shoaib et al., 2016). The SCFA catabolites generated upon the carbohydrate fermentation were also measured during the bacterial growth. This study is expected to contribute to the improvement of the efficiency of the targeted biocatalytic approach for the feruloylation of polysaccharides and to the understanding of the effect of feruloylation on the prebiotic property.

6.3. Materials and methods

6.3.1. Materials

Stevens variety cranberries were provided by Les Canneberges Atoka Inc., Canada and stored at -20 °C. Adsorbent beads Sepabeads® EC-EP R and Relizyme® EP403 R were obtained from Resindion S.R.L (Binasco, Italy). Feruloyl esterase from *H. insolens* was obtained from Biocatalysts Ltd. (Cardiff, UK). HPLC grade solvents including formic acid, acetonitrile, n-hexane and butanone were purchased from Fisher Scientific (Waltham. MA, USA) and analytical grade reagents including premixed MRS and reinforced clostridial media, agar and Tween 80 were purchased from Sigma-Aldrich Canada Co. (St. Louis, MO, USA). Bacterial strains *Lactobacillus brevis* (ATCC® 14869™) and *Bifidobacterium longum* subsp. *longum* (ATCC® 15707™) were obtained from Cedarlane (Burlington, Canada).

6.3.2. Isolation of cell wall polysaccharides from cranberry pomace

Chelating agent extract (CH) and diluted alkali extract (DA) enriched with pectic polysaccharides were obtained and characterized. Briefly, Stevens variety cranberries were defrosted, blended, and pressed in cheese cloth. The remaining pomace was then freeze dried and blended into a fine powder. Alcohol insoluble solids were obtained by suspending the powder in 95% ethanol at a concentration of 13.8 % (w/v) using gentle shaking. The recovered alcohol insoluble solids were washed three times with 85% ethanol, chloroform:methanol (1:1, v:v), and acetone then dried overnight at room temperature. The CH extract was obtained by suspending the dry alcohol insoluble solids (2.66%, w/v) in 0.05 M sodium acetate buffer at pH 5.2 containing 0.05 M ethylenediaminetetraacetic acid (EDTA) and 0.05 M sodium oxalate. The recovered solids were then suspended in 0.05 M sodium hydroxide (2.66%, w/v) to yield DA extract. Extracts were dialyzed (cut-off 5-8 kDa), freeze dried and characterized in terms of molecular weight distribution, the monosaccharide profile and the total phenolic content.

6.3.3. Immobilization of feruloyl esterase

Prior to immobilization, epoxy-activated supports were modified by suspending them (0.2 g/l) in a solution of 1.8 M iminodiacetic acid (IDA) brought to alkaline pH with sodium hydroxide (pH of 9 for Sepabeads® EC-EP R, pH of 11 for Relizyme® EP403 R) and shaken for 5 h at 55 rpm in Excella E24 orbital shaker (New Brunswick Scientific, Edison, NJ, USA) at room temperature. Excess reagents were removed by washing the supports (0.2 g/l) eight times with water. After each wash the supports were vacuum filtered on a fritted glass filter. The supports were then suspended (0.17 g/l) in cupric sulfate (0.13 M for Sepabeads® EC-EP R, 0.2 M for Relizyme® EP403 R) for 2 h under the same conditions as the previous step. Activated supports were then washed (0.2 g/l) eight times in water and five times in 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (0.02 M, pH of 6) and stored in MOPS buffer at 4 °C. The amount of IDA and copper bound to the supports were determined respectively by titration and by measuring the changes in the absorbance at 750 nm of the copper sulfate solutions.

Modified IDA-Cu-Sepabeads® EC-EP R support was washed with 0.2 M potassium phosphate buffer at pH 8, while modified IDA-Cu-Relizyme® EP403 R support was washed with 0.86 M potassium phosphate buffer at pH of 5, according to the optimized conditions for immobilization determined in our previous works (Tamayo-Cabezas & Karboune, 2019). The supports were then suspended (0.2 g/l) in the same buffers containing 0.01 g (IDA-Cu-Sepabeads® EC-EP R) or 0.02 g (IDA-Cu-Relizyme® EP403 R) of enzyme per gram of support. The suspensions were kept for 28 to 36 h at 6 °C under gentle shaking. Supernatants were recovered by centrifugation at 2400 × g for 5 min, and the supports, containing immobilized enzyme, were washed (0.2 g/l) five times with MOPS buffer (0.02 M, pH of 6) and stored at 4 °C. Immobilization blanks were prepared by incubating the enzyme solutions in the absence of support. Protein contents of the initial enzyme solutions, the supernatants and the washing solutions were measured by Bradford assay. Hydrolytic activities of the initial enzyme suspension, the immobilization blanks, the supernatants and of the washing solutions were determined using methyl ferulate substrate as described by Tamayo-Cabezas and Karboune (2019). Immobilization yield was calculated as the difference between activity in the immobilization blank and the activities in supernatant and washing solutions, divided by the activity of the initial enzyme solution.

6.3.4. Esterification activity of free and immobilized enzyme

The esterification activity of feruloyl esterase was assessed using raffinose and ferulic acid as substrates at a concentration of 1.5 and 4.5 mM, respectively.

A surfactantless microemulsion made of n-hexane:butanone:water was evaluated as a reaction medium at selected ratios of 51:46:3, 51.8:46.7:1.5 and 49.4:44.6:6 (v:v:v). In vacuum sealed flasks, 10 g/l of free or immobilized feruloyl esterase were added to each emulsion. The reaction mixtures were incubated for 24 h at 35 °C under 70 rpm. Negative controls without enzyme and without raffinose were run in parallel. Reaction progress was monitored through the consumption of ferulic acid, determined by high performance liquid chromatography (HPLC) on a Waters Breeze 2 system (Waters Corp., Milford, MA) equipped with a binary pump (Model 1525), and a photodiode array detector (Model 2998). The reaction mixture was loaded on a Zorbax SB-C18 reversed-phase column (5 µm, 250×4.6 mm, Agilent Technologies Canada Inc.; Mississauga, Canada) and eluted with a linear gradient from mobile A (80% of formic acid (5.5%, v/v) in water and 20% acetonitrile) to mobile B (100% acetonitrile) for 30 min. A constant flow rate of 0.7 mL /min was applied, and the absorbance was continually monitored at 320 nm. Ferulic acid standard was used to construct the calibration curve. One unit of esterification activity was defined as the amount of enzyme that consumes one micromole of ferulic acid per minute.

6.3.5. Feruloylation of polysaccharides

The feruloylation of cranberry polysaccharides was carried in the n-hexane:butanone:water surfactantless microemulsion. The appropriate ratio of n-hexane:butanone:water reaction medium was used for each reaction system (free and immobilized feruloyl esterase on IDA-Cu-Sepabeads® and IDA-Cu-EC-EP R supports) based on the esterification activity on raffinose. The enzymatic feruloylation reaction was initiated by adding the free or immobilized feruloyl esterase (1 U/l) into the microemulsions containing CH or DA (0.3 g/l) polysaccharide extract and ferulic acid (1.5 g/l). The enzymatic reactions were conducted in vacuum sealed flasks at 35 °C for 144 h, with a continuous shaking at 70 rpm. The mixtures were then centrifuged at 2400 × g for 3 min and the esterified polysaccharides (FA-CH, FA-DA) isolated from the supernatants by extraction with water. The aqueous phase was dialyzed (cut-off 5-8 kDa) and freeze dried. Reaction yield was calculated from the decrease of ferulic acid concentration in the reaction supernatants, measured by HPLC as described in par. 6.3.4.

6.3.6. Analytical methods for quantification and characterization of polysaccharides

6.3.6.1. Monosaccharide composition

To determine the monosaccharide composition, the polysaccharides present in the CH and DA extracts were first hydrolyzed using a two-step procedure as previously described by Khodaei & Karboune (2013).

The samples were suspended and incubated at 60 °C for 24 h in HCl/methanol mixture (1:4, v/v) at a ratio of 0.6% (w/v) and thereafter boiled for 1 h in trifluoroacetic acid solution at a ratio of 1:8 (v/v). Hydrolyzed samples were analyzed with high performance anionic exchange chromatography, equipped with pulsed amperometric detector (ICS 3000, Dionex Co., Sunnyvale, CA, USA), and a CarboPac PA20 column (3×150 mm). Isocratic elution was performed with 5 mM NaOH (0.5 ml/min). L-Rhamnose (Rha), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-xylose (Xyl) and D-mannose (Man) were used as standards.

6.3.6.2. Molecular weight profile

A high-performance size-exclusion chromatography (HPSEC) system (Model 1525, Waters Co., Milford, MA, USA) was used to characterize the molecular weight distribution. Columns TSK G5000 PWXL and TSK G3000 PWXL (Tosoh Co, Yamaguchi, Japan) were used in series with isocratic flow rate of 0.4 mL /min of 0.1 M sodium chloride. Refractive index detector was operated at 30 °C. Dextrans (50-670 kDa) standards (0.3 - 5 g/l) were used for calibration.

6.3.6.3. Phenolic content

Native and feruloylated polysaccharides were characterized in terms of total phenolic content by Folin-Ciocalteu colorimetric method (Singleton et al., 1999). To 0.8 mL of aqueous solution of the sample, 0.15 mL of sodium bicarbonate (250 g/l) and 0.05 ml of Folin-Ciocalteu reagent were added. The mixtures were heated at 40 °C for 30 min, and their absorbance was measured at 765 nm. Gallic acid standard solutions (0.001 M to 0.1 M) were used for calibration.

6.3.7. Prebiotic activity assay

Cranberry polysaccharides (CH, DA extracts) as well as corresponding feruloylated polysaccharides (FA-CH, FA-DA) were investigated as a carbon source for *B. longum* and *L. brevis* growth compared to growth on inulin. *B. longum* and *L. brevis* were maintained in anaerobic chamber in an atmosphere composed of 5% hydrogen, 5% carbon dioxide and 90% nitrogen, at 37 °C. Pre-reduced reinforced clostridial broth was used for reanimation of *B. longum* from a freeze dried stock, and MRS broth was used for *L. brevis*. An aliquot (0.5 mL) of the primary cultures was then added into tubes containing 5 mL of pre-reduced, carbohydrate-free MRS broth, supplemented with 0.4% w/v glucose. Bacterial growth was measured by colony count on pre-reduced MRS agar.

Tubes containing 5 mL of pre-reduced, carbohydrate-free MRS broth supplemented with 0.2% w/v of glucose, inulin, CH, DA, FA-CH, or FA-DA were inoculated with 0.1 mL of secondary culture. Colonies were counted on pre-reduced MRS agar plates after 48 and 96 h of incubation. A prebiotic activity score (PA) was calculated for each combination of bacterium and polysaccharide as follows: PA = (log CFU/ml on polysaccharide at 96 h – log CFU/ml on polysaccharide at 0 h)/(log CFU/ml on glucose at 96 h – log CFU/ml on glucose at 0 h).

6.3.8. Determination of short chain fatty acids catabolites

An aliquot of cultures (0.5 mL) was taken at 0, 48 and 96 h. Bacteria were precipitated by centrifugation at 5000 rpm for 5 min in a Minispin Plus centrifuge (Eppendorf AG, Hamburg, Germany). The supernatants were filtered through PTFE syringe filter of pore size 0.45 µm and stored at -20 °C. Freshly thawed samples (0.02 ml) were analyzed by HPLC on a Model 1525 system (Waters Co., Milford, MA, USA) equipped with a refraction index detector and a Hypersil GOLD C18 column (250 mm × 4.6 mm) (Thermo Scientific, U.S.A). Elution was performed with 0.005 N sulfuric acid at constant flow rate 0.6 ml/min. Standards were prepared by serial dilution of a stock solution of lactic acid, acetic acid, propionic acid and butyric acid in water.

6.3.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.4.2 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance (ANOVA), Tukey's honest significant difference test and Mann-Whitney test were performed to detect significant differences ($P < 0.05$).

6.4. Results and discussion

6.4.1. Feruloyl esterase immobilization

The modification of supports and the immobilization of feruloyl esterase are reported in Table 6.1. More IDA, copper ions and enzyme were bound on Relizyme® EP403 R compared to Sepabeads® EC-EP R. These differences can be attributed to the larger pore size of Relizyme® EP403 R support that may have allowed a higher interaction of the epoxy groups with the reactants and offered less hindrance to the access of the enzyme to the active immobilization sites on the support.

Table 6.1 Modification of epoxy-activated supports and immobilization of feruloyl esterase.

	Sepabeads® EC-EP R	Relizyme® EP403 R
Modified supports		
- Minimum epoxy group density[a] (10^{-6} mol/g)	200	100
- Pore diameter[a] (nm)	10-20	40-60
- Bound IDA (10^{-6} mol/g beads)	11.9 ± 1.1	47.8 ± 0.9
- Bound copper (10^{-6} mol/g beads)	65.8 ± 5.4	200.5 ± 31.4
Immobilized feruloyl esterase		
- Enzyme amount (mg/ g beads)	0.8 ± 0.2	1.7 ± 0.1
- Immobilization yield % w/w	72.5 ± 12.7	90.1 ± 9.8

All values are expressed as mean ± SD.

IDA: Iminodiacetic acid.

[a]: Data from manufacturer.

Similar results were reported for the immobilization of feruloyl esterase-containing enzymatic mixture by adsorption on mesoporous silica; as the support with larger pore size (9 nm) was found to have greater immobilization yield than the one with smaller (5 nm) pores (Thörn et al., 2011). Immobilization yield on IDA-Cu-Sepabeads® EC-EP R was similar to the one previously reported (66.2 - 71.7%) for the immobilization of feruloyl esterase on selected epoxy-based supports (Tamayo-Cabezas & Karboune, 2019).

Table 6.2 shows the esterification activity of free and immobilized feruloyl esterase from *H. insolens* for the feruloylation of raffinose in three selected surfactantless microemulsion systems. Free feruloyl esterase was found to have significantly higher ($P<0.05$) activity in the reaction medium with the highest percentage of water (6%). This reveals that the presence of water positively affected the catalytic activity of free feruloyl esterase, possibly by allowing the formation of a hydration shell that preserves the enzyme in active configuration (Yang et al., 2004). In contrast, variation of water concentration in the range of 10-13% was reported not to affect the feruloylation of 1-pentanol catalyzed by free *Aspergillus niger* feruloyl esterase in a similar microemulsion system (Giuliani et al., 2001). While a feruloyl esterase from *Fusarium oxysporum* was found to promote the esterification of 1-propanol with p-hydroxyphenylacetic acid in the presence of a lower water proportion (2%) in the microemulsion (Topakas et al., 2003).

Compared to other biocatalysts, feruloyl esterase immobilized on IDA-Cu-Relizyme® EP403 R had the lowest specific activity and led to a detectable consumption of ferulic acid in only the microemulsion reaction medium with 3% water content. Compared to it, feruloyl esterase immobilized on IDA-Cu-Sepabeads® EC-EP R exhibited a higher specific activity in the surfactantless microemulsion reaction media with the two highest contents of water (3 and 6%, v/v), indicating that IDA-Cu-Sepabeads® EC-EP R is best suited as an immobilization support for the glycoside feruloylation application. Both immobilized feruloyl esterase biocatalysts were found to have a maximum esterification activity at 3% water content. These results reveal that in the presence of immobilization support, a microemulsion reaction medium with 3% water content led to a good compromise between the denaturing effect of low-water environment and the promotion of the competing ester hydrolysis activity caused by water. While higher amount of water of 6% in the microemulsion reaction medium is needed to achieve the maximal esterification activity of free feruloyl esterase.

Table 6.2 Specific raffinose esterification activity (mmol of ferulic acid consumed per min per mg of catalyst) of free and immobilized feruloyl esterase in selected microemulsion systems.

	n-hexane: butanone: water (%v/v)		
Biocatalyst	51.8: 46.7: 1.5	51: 46: 3	49.4: 44.6: 6
Free enzyme	74.2 ± 1.3 (0.6)	60.9 ± 1.4 (0.5)	140.7 ± 37.8 (1.2)
On IDA-Cu-Sepabeads® EC-EP R	n.d.	100.2 ± 18.9 (125.3)	41.8 ± 0.9 (52.3)
On IDA-Cu-Relizyme® EP403 R	n.d.	29.4 ± 11.5 (17.3)	n.d.

All values are expressed as mean ± SD. n.d.: not detected.

Values in brackets represent micromoles of ferulic acid consumed per min per 10^{-3} mg of protein, as determined by colorimetric test.

The difference between free and immobilized feruloyl esterase can be attributed to the micro-environment effect promoted in the heterogeneous reaction system and/or to the aggregation of free enzyme in non-conventional reaction system limiting access to water. Noticeably, at 3% water content, the esterification specific activity of feruloyl esterase immobilized on IDA-Cu-Sepabeads® EC-EP R was found to be significantly higher ($P<0.05$) than the one of free enzyme, indicating that immobilization on this support significantly promoted the esterification activity at the investigated conditions. This may be due to the fact that immobilization led to a stabilizing effect on the enzyme's tertiary structure, reducing the denaturing effect of low-water environment.

6.4.2. Feruloylation of cranberry pectic polysaccharide extracts

Two polysaccharide extracts, CH and DA, were isolated from cranberry pomace and analysed. As shown in Table 6.3, CH polysaccharide extract contained three main populations of poly- and oligosaccharides with different molecular weight distributions (1800, 100, 1.5 kDa), while DA polysaccharides fit into one main population (320 kDa). Both extracts contained mainly pectic polysaccharides, as uronic acid dominated the monosaccharide profile. The presence of homogalacturonan (polygalacturonic acid) appears to be more abundant in CH than in DA. From the profile of neutral monosaccharides, it is possible to estimate the abundance of other structures, whose presence was confirmed by glycosidic linkage analysis. In addition to homogalacturonan, CH contains 12.8% rhamnogalacturonan type I and 4.9% hemicellulose, while DA appears to contain 23.2% rhamnogalacturonan type I and 3.3% hemicellulose. Both extracts contain small amounts of phenolics, attributable to the presence of pectic polysaccharide-bound compounds such as proanthocyanidins, that also occur in pectin-rich extracts of grapes (Ruiz-Garcia et al., 2014).

The feruloylation of CH and DA polysaccharides with free and immobilized feruloyl esterase was investigated using 49.4:44.6:6 and 51:46:3 (% v/v) of n-hexane:butanone:water surfactantless microemulsion reaction medium, respectively. The appropriate reaction medium for each biocatalyst was identified upon the esterification of raffinose with ferulic acid (Table 6.2), as discussed previously. As shown in Table 6.4, free and immobilized feruloly esterase performed similarly on DA, resulting in the feruloylation extent of 18.0 to 22.9%. While, using CH as substrate, immobilized feruloyl esterase led to higher feruloylation (22.1 to 26.6%) than the free enzyme (4.2%) and yielded polysaccharides with higher total phenolic content (1.40×10^{-3} – 1.74×10^{-3} moles of gallic acid per gram of solid).

Table 6.3 Characterization of cranberry chelating agent (CH) and diluted alkaline (DA) polysaccharide extracts.

	CH	DA
Molecular weight (10^3 Da)	1800; 100; 1.5	320
Total phenolic content (10^{-6} mol GA/g)	31.9 ± 6.4	80.8 ± 6.6
Monosaccharide profile (%mol)		
Rhamnose	1.1 ± 0.1[a]	1.6 ± 0.1[a]
Arabinose	7.1 ± 0.1[a]	13.8 ± 0.4[a]
Galactose	3.5 ± 0.7[b]	6.2 ± 0.3[a]
Glucose	2.8 ± 0.1[a]	1.7 ± 0.9[a]
Mannose, Xylose	2.1 ± 0.1[a]	1.6 ± 0.6[a]
Uronic acids	83.4 ± 0.4[a]	75.2 ± 0.3[b]

Values represented as average ± standard deviation.

CH: Chelating agents extract; DA: diluted alkali extract; GA: gallic acid.

[a-b]: Within the same row, means with different letters are significantly different at $P \leq 0.05$.

Table 6.4 Feruloylation extent and increase in total phenolics content of polysaccharides present in the cranberry chelating agent extract (CH) and diluted alkali extract (DA) upon esterification reaction (144 h) catalyzed by free and immobilized feruloyl esterase.

	Feruloylation extent (% mol) [a]		Total phenolics increase (10^{-3} mol gallic acid/g extract) [b]	
Catalyst (1 U/l)	CH	DA	CH	DA
Free enzyme	4.2 ± 3.6[d]	18.9 ± 0.0[c]	0.35 ± 0.04[e]	1.32 ± 0.02[c]
On modified Sepabeads® EC-EP R	26.6 ± 6.9[c]	22.9 ± 6.6[c]	1.74 ± 0.03[c]	1.59 ± 0.11[c]
On modified Relizyme® EP403 R	22.1 ± 2.6[c]	18.0 ± 0.2[c]	1.40 ± 0.04[d]	1.33 ± 0.10[c]

All values are expressed as mean ± SD.

CH: Chelating agent extract; DA: diluted alkali extract.

[a] Calculated as the difference between moles of ferulic acid in blanks and in final reaction mixtures.

[b] Calculated as the difference between moles of phenolic compounds (gallic acid equivalent) per gram of dry extract after enzymatic treatment and before treatment.

[c-e] Within the same column, means with different letters are significantly different at $P \leq 0.05$.

This indicates that immobilized feruloyl esterase is more suitable for the feruloylation of CH polysaccharides than the free one.

To the authors' knowledge, this is the first study on the feruloylation of polysaccharides by feruloyl esterase. The observed feruloylation extents were within the same range as those reported for analogous reaction systems on xylobiose (27.4%) catalyzed by *H. insolens* feruloyl esterase (Tamayo-Cabezas & Karboune, 2019) and on L-arabinobiose (10 to 18%) catalyzed by *Sporotrichum thermophile* feruloyl esterase (Vafiadi et al., 2006). The feruloylation extent of mono, di and oligosaccharides was found to be dependent on the structural characteristics of glycosides, in particular the type of hexose/pentose moiety, the length and the glycosidic linkages (Couto et al., 2011; Tamayo-Cabezas & Karboune, 2019).

In the present study, the differences in size and composition between polysaccharides had limited impact on the feruloylation. Indeed, comparing CH and DA extracts, no significant difference in the feruloylation or in the increase of phenolic content was observed. A naturally occurring pectin feruloylation was described as being mainly localized on rhamnogalacturonan type I neutral branches, in particular at the O2 or O3-position of arabinans and the O6-position of galactans (Holck et al., 2011; Ralet et al., 1994; Sato et al., 2013). To the best of our knowledge, it has not been investigated if any of the known feruloyl esterases could have activity towards the galacturonic acid residues of the homogalacturonan. While feruloyl esterases have been described as able to hydrolyze the ester bond formed between the carboxylic acid of uronic residues and the hydroxyl groups of lignin (Mäkelä et al., 2015), little evidence supports this statement. Since enzyme immobilized on IDA-Cu-Sepabeads® EC-EP R generated a CH extract with higher ($P<0.05$) content of ferulic acid, this catalyst was deemed the best of the three for the generation of feruloylated polysaccharides at the tested conditions.

6.4.3. Prebiotic activity

The prebiotic activity scores of native and feruloylated polysaccharides and of inulin, a known prebiotic polysaccharide (Shoaib et al., 2016) were compared by using them as carbon sources for the anaerobic growth of two probiotic bacteria strains. Both strains were handled and grown in anaerobic conditions. While *L. brevis* is oxygen-tolerant, the anaerobic conditions were selected to simulate more accurately the intestinal environment. The results, shown in Figure 6.1, indicate that the investigated polysaccharides were suitable carbon sources for both bacteria, as growth was observed. It is important to note that CH and DA extracts contained a combination of polygalacturonic acid, branched rhamnogalacturonan I and non-pectic structures (Table 6.3).

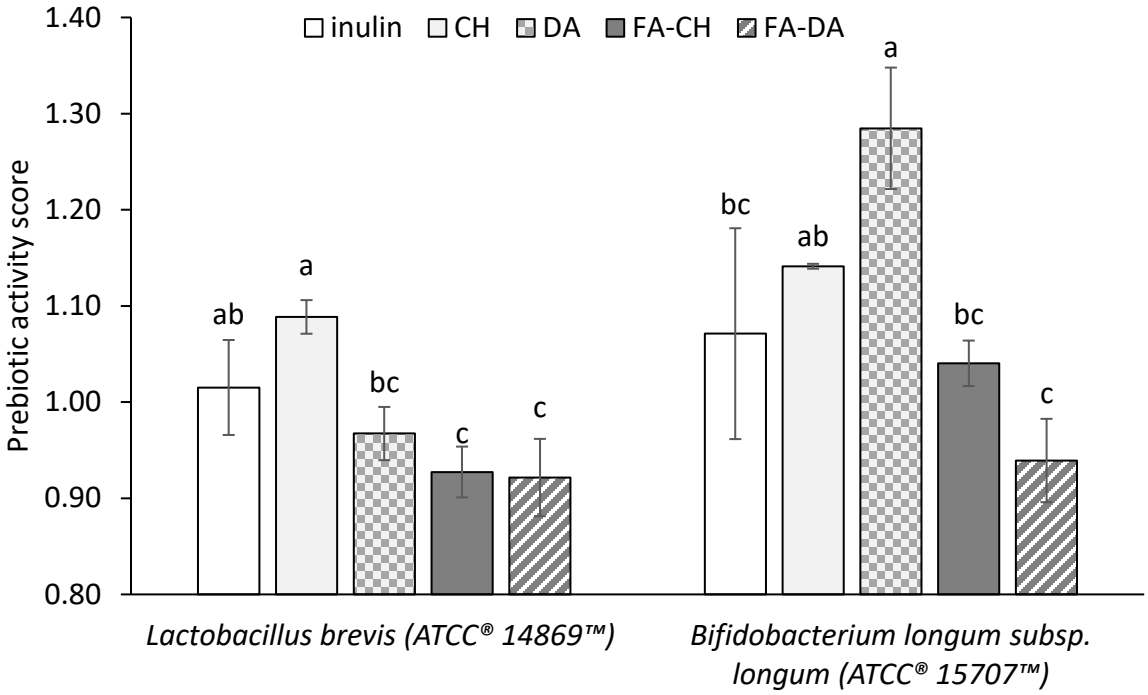

Figure 6.1 Prebiotic activity scores of different bacteria on polysaccharide extracts, feruloylated extracts and inulin, measured at 96 h of incubation. Values represented as average ± standard deviation. CH: chelating agent extract; DA: diluted alkali extract; FA-CH and FA-DA: feruloylated CH and DA. For each bacterial strain, bars with different letters represent scores significantly different at $P \leq 0.05$.

Tingirikari (2019) reported that sugar beet arabinan, larch arabinogalactan and soy rhamnogalacturonan, but not citrus polygalacturonic acid, promoted the growth of *B. longum*, while none promoted the growth of *L. brevis* in an aerobic environment. A combination of polysaccharides seems therefore to be more appropriate as a carbon source for *L. brevis,* probably due to how its carbohydrate catabolism pathways are regulated. In general, *Lactobacillus* species exhibited a significant heterogeneity in their abilities to ferment carbohydrates (Boguta et al., 2014; Broadbent et al., 2012; Kim et al., 2010).

CH polysaccharides showed a prebiotic activity score similar to that of inulin for *L. brevis*, and significantly higher ($P \leq 0.05$) than that of FA-CH. For *B. longum*, DA polysaccharides had the highest prebiotic activity score, significantly higher than both FA-DA polysaccharides and inulin. Similarly, CH polysaccharides showed a higher prebiotic activity score for *L. brevis* than FA-CH ones. Therefore, it appears that increased content of ferulic acid had a negative impact on the growth of both strains. This impact is more pronounced for *B. longum* and DA polysaccharides rich in rhamnogalacturonan I. Esterification with up to 247.3×10^{-6} moles of ferulic acid per gram of dry sugar beet pectic oligosaccharides was previously found to not significantly affect the prebiotic activity (Holck et al., 2011). The analysis of wheat bran xylo-oligosaccharides showed that the presence of ferulic acid (202.1×10^{-6} mol/g) modulated their prebiotic properties (Gong et al., 2019). As the phenolic content of FA-CH and FA-DA polysaccharides was on average much higher (1719.9×10^{-6} mol/g), it can be assumed that additional feruloylation poses an obstacle to the fermentation of these polysaccharides by *B. longum* and *L. brevis.* Probiotic bacteria, such as *Lactobacillus* spp. and *Bifidobacterium* spp., have shown to produce feruloyl esterase to release ferulic acid from arabinoxylan-oligosaccharides (Ou & Sun, 2014; Szwajgier & Jakubczyk, 2010). However, an excess of ferulic acid may have inhibited the endogenous glycosyl-enzymes expressed by probiotic bacteria (Boukari et al., 2011; Rohn et al., 2002). Further investigation with more strains of probiotic and non-probiotic bacteria is needed to assess whether these detrimental effects of the feruloylation equally affect all microbiota or could result in the differential promotion of the growth of selected bacteria.

6.4.4. Short chain fatty acids profile

SCFA are produced upon the fermentation of dietary fiber by gut microbiota (Lin et al., 2011). Tables 6.5 and 6.6 show the concentration of four SCFA after 48 and 96 h of fermentation of glucose, inulin, native and feruloylated polysaccharides.

In the culture of *L. brevis* (Table 6.5), the main increase was seen in the propionic acid followed by acetic acid, while the concentration of lactic acid and butyric acid only showed small changes over fermentation time. Pectic oligosaccharides have been reported to generate primarily acetic acid, followed by propionic and butyric acids when fermented *in vitro* by fecal microorganisms (Di et al., 2017; Dongowski & Lorenz, 1998). While lactic and formic acids were initially produced but got degraded as the fermentation continued.

The different behavior in the present study can be attributed to the use of a single bacteria strain, possessing utilization pathway that diverges from the average consumption of the whole microbiota. While *Lactobacillus* genus is more known for its lactic acid fermentation, NCBI protein database shows that *L. brevis* possesses key enzymes for the fermentation of rhamnose to propionic acid, such as a putative lactaldehyde dehydrogenase, propanediol dehydratase and propanal dehydrogenase (NCBI Resource Coordinators, 2018). The selected growth conditions that favor this pathway remain to be clarified. DA polysaccharides were associated to the production of acetic acid by *L. brevis* at a level comparable with glucose utilization at 48 h; while its fermentation of other polysaccharides reached comparable levels of acetic acid only at 96 h. Fermentation of CH, DA, FA-CH and FA-DA by *L. brevis* displayed identical patterns for the production of lactic and butyric acid; while differences could be observed in the profiles of acetic and propionic acid. Growth on DA liberated more acetic acid than on CH at the first time point, and the same held true for FA-DA compared to FA-CH. This is possibly due to the fact that *L. brevis* may have favored the utilization of neutral sugar branches, of which DA is richer. As at 96 h, these differences disappeared and total SCFA generated by *L. brevis* were higher with CH and FA-CH than with their DA counterparts. It appears that polysaccharides in DA were more rapidly fermented into acetic acid than those in CH, but this didn't affect fermentation in the long-term.

The use of FA-DA polysaccharides by *L. brevis* was associated with the lowest level of propionic acid at both fermentation times. While its growth on FA-CH polysaccharides initially generated an amount of propionic acid comparable to the other extracts, the amount at longer fermentation time was found to be inferior to that of CH. These observations point towards feruloylation being the main factor negatively affecting propionic acid production by *L. brevis*. The profile of propionic acid production at 96 h matched with the probiotic activity score on *L. brevis*, as the polysaccharides that had higher prebiotic activity score also produced higher concentration of propionic acid. Thus, propionic acid concentration appeared as a valid indicator of bacterial growth promotion for this strain.

Table 6.5 Concentration (10^{-3} mol/l) of short chain fatty acids released in the samples obtained from the *Lactobacillus brevis* (ATCC® 14869™) fermentation using different carbon sources. Values determined as difference with the concentrations detected immediately after inoculation.

Lactic acid

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	23.2 ± 6.7^{a}	3.4 ± 0.6^{b}	3.9 ± 0.5^{b}	3.4 ± 0.2^{b}	5.1 ± 0.7^{b}	4.4 ± 1.0^{b}
96	26.8 ± 5.8^{a}	5.2 ± 0.9^{b}	7.1 ± 0.2^{b}	5.6 ± 0.8^{b}	5.2 ± 0.2^{b}	3.9 ± 1.0^{b}

Acetic acid

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	15.7 ± 0.0^{a}	3.1 ± 1.0^{b}	2.5 ± 0.0^{b}	n.d.	14.0 ± 0.6^{a}	7.2 ± 2.4^{ab}
96	17.0 ± 7.9^{a}	12.4 ± 4.3^{a}	18.5 ± 0.7^{a}	10.3 ± 6.3^{a}	10.8 ± 2.1^{a}	11.2 ± 3.0^{a}

Propionic acid

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	n.d.	23.8 ± 3.7^{a}	13.6 ± 4.1^{b}	15.9 ± 6.6^{ab}	9.6 ± 5.5^{b}	n.d.
96	46.3 ± 2.0^{ab}	54.1 ± 8.1^{a}	53.1 ± 8.4^{a}	31.8 ± 6.2^{c}	39.2 ± 5.2^{bc}	9.3 ± 1.2^{d}

Butyric acid

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	0.6 ± 0.1^{a}	0.3 ± 0.2^{a}	0.4 ± 0.0^{a}	0.6 ± 0.6^{a}	0.5 ± 0.1^{a}	0.2 ± 0.1^{a}
96	0.5 ± 0.1^{a}	0.5 ± 0.3^{a}	0.2 ± 0.1^{a}	0.8 ± 0.2^{a}	0.3 ± 0.2^{a}	0.6 ± 0.1^{a}

Total short chain fatty acids

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	39.4 ± 3.9^{a}	30.5 ± 2.3^{ab}	20.4 ± 2.9^{bc}	19.9 ± 4.7^{bc}	29.2 ± 3.9^{ab}	11.7 ± 1.8^{c}
96	90.6 ± 6.7^{a}	72.1 ± 5.3^{b}	78.8 ± 5.9^{ab}	48.5 ± 5.7^{c}	55.4 ± 4.0^{c}	25.1 ± 2.4^{d}

Values represented as average ± standard deviation.

CH: Chelating agents extract; FA-CH: feruloylated CH; DA: diluted alkali extract; FA-DA: feruloylated DA.

$^{a-d}$: Within the same row, means with different letters are significantly different at $P \leq 0.05$.

The general profile of SCFA in the culture of *B. longum* (Table 6.6), similarly to the one of *L. brevis*, was characterized by the greatest increase in propionic and acetic acid. All investigated polysaccharide carbon sources caused a small increase of lactic acid at 48 h that remained almost unchanged at the later time point without affecting the butyric acid content of the medium. Polysaccharides had different profiles of SCFA production by *B. longum* over fermentation time. A significant difference was identified at 48 h, at which CH fermentation yielded more propionic acid than all other carbon sources, suggesting that the properties of CH polysaccharides made it more readily accessible to the *B. longum* strain. However, this difference was less marked at 96 h, as the use of DA polysaccharides caused increase of propionic acid between 48 and 96 h when fermented by *B. longum*. Finally, all polysaccharides were associated to the generation of less lactic acid than fermentation of glucose, a trait observed also for the fermentation by *L. brevis*. Similarly to what was seen with *L. brevis*, it was possible to directly correlate the total amount of propionic acid produced by *B. longum* at 96 h of growth with the prebiotic activity score of various polysaccharides, with the two feruloylated ones performing similarly to each other and less than the native ones, the only difference being that inulin yielded an amount of acid comparable to DA polysaccharides, while the prebiotic activity score was lower. Overall, comparing the total SCFA released at 96 h revealed that the presence of feruloylation affected DA more than CH. Considering the similar phenolic contents of FA-DA and FA-CH, this difference may derive from the interaction that the ferulic esters may have had with the more abundant neutral sugar residues of DA. A difference emerges between the two bacteria, with the fermentation by *L. brevis* being more affected by feruloylation than the one by *B. longum*.

Overall, comparing the total SCFA released at 96 h revealed that the presence of feruloylation affected DA more than CH. Considering the similar phenolic contents of FA-DA and FA-CH, this difference may derive from the interaction that the ferulic esters may have had with the more abundant neutral sugar residues of DA. A difference emerges between the two bacteria, with the fermentation by *L. brevis* being more affected by feruloylation than the one by *B. longum*.

Table 6.6 Concentration (10^{-3} mol/l) of short chain fatty acids in the samples obtained from *Bifidobacterium longum* subsp. *longum* (ATCC® 15707™) fermentation using different carbon sources. Values determined as difference with the concentrations detected immediately after inoculation.

Lactic acid

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	18.5 ± 0.9^{a}	4.2 ± 1.4^{b}	5.0 ± 1.3^{b}	4.2 ± 0.6^{b}	2.0 ± 0.7^{b}	3.3 ± 0.8^{b}
96	24.9 ± 0.9^{a}	5.8 ± 1.0^{b}	4.7 ± 1.4^{b}	3.5 ± 1.2^{b}	2.6 ± 2.6^{b}	2.4 ± 2.2^{b}

Acetic acid

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	n.d.	n.d.	6.5 ± 0.9^{a}	3.3 ± 0.3^{a}	10.0 ± 0.9^{a}	3.1 ± 2.7^{a}
96	8.0 ± 1.4^{a}	15.5 ± 5.4^{a}	16 ± 3.1^{a}	5.8 ± 0.0^{a}	18.2 ± 0.9^{a}	9.5 ± 4.2^{a}

Propionic acid

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	1.5 ± 0.7^{c}	6.8 ± 0.0^{b}	22.3 ± 0.8^{a}	0.6 ± 0.0^{c}	n.d.	1.3 ± 0.4^{c}
96	37.2 ± 6.1^{a}	34.3 ± 14.3^{a}	28.8 ± 10.6^{ab}	23.2 ± 5.0^{bc}	33.7 ± 6.6^{a}	13.4 ± 5.3^{c}

Butyric acid

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	0.1 ± 0.0^{a}	n.d.	0.5 ± 0.0^{a}	n.d.	0.1 ± 0.0^{a}	n.d.
96	n.d.	0.7 ± 0.2^{a}	0.6 ± 0.3^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.6 ± 0.0^{a}

Total short chain fatty acids

Time (h)	Glucose	Inulin	CH	FA-CH	DA	FA-DA
48	20.1 ± 0.7^{b}	11.0 ± 0.8^{b}	34.3 ± 1.0^{a}	8.2 ± 0.4^{b}	12.0 ± 0.7^{b}	7.7 ± 2.0^{b}
96	70.1 ± 3.7^{a}	56.2 ± 8.8^{b}	50.1 ± 6.5^{b}	32.7 ± 3.6^{c}	54.7 ± 4.1^{b}	25.9 ± 4.6^{c}

Values represented as average ± standard deviation.

CH: Chelating agents extract; FA-CH: feruloylated CH; DA: diluted alkali extract; FA-DA: feruloylated DA.

$^{a-c}$: Within the same row, means with different letters are significantly different at $P \leq 0.05$.

6.5. Conclusions

H. insolens feruloyl esterase, immobilized on iminodiacetic acid-copper modified epoxy-activated supports, proved to be suitable for the esterification of cranberry pectic polysaccharides (CH and DA) with ferulic acid in a microemulsion medium made of n-hexane:butanone:water of 51:46:3 (%v/v), resulting in feruloylation extent of 22.1-26.6% over a reaction time of 144 h. When tested for the promotion of the growth of selected probiotic strains, the feruloylated polysaccharides performed similarly or less than the non-feruloylated ones and inulin, with DA polysaccharides fermentation being affected the most by feruloylation. Short chain fatty acid profiles were characterized by high amounts of propionic acid, followed by acetic acid, lactic acid and butyric acid. Bacterial growth on the polysaccharides found direct correlation with the development of short chain fatty acids, and in particular of propionic acid in the culture of *B. longum* and *L. brevis*. The effect of feruloylation was more marked on the fermentation by *L. brevis.* Further investigation of the utilization of these polysaccharides by other bacteria and by the intestinal microbiota is needed to assess the effect of feruloylation on the selectivity of colonic fermentation.

6.6. References

Al-Lahham, S. H., Peppelenbosch, M. P., Roelofsen, H., Vonk, R. J., & Venema, K. (2010). "Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms". Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids *1801*(11): 1175–1183.

Boguta, A. M., Bringel, F., Martinussen, J., & Jensen, P. R. (2014). "Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks". Microbial Cell Factories *13*(1): 97.

Boukari, I., O'Donohue, M., Rémond, C., & Chabbert, B. (2011). "Probing a family GH11 endo-β-1,4-xylanase inhibition mechanism by phenolic compounds: Role of functional phenolic groups". Journal of Molecular Catalysis B: Enzymatic *72*(3–4): 130–138.

Broadbent, J. R., Neeno-Eckwall, E. C., Stahl, B., Tandee, K., Cai, H., Morovic, W., … Steele, J. L. (2012). "Analysis of the Lactobacillus casei supragenome and its influence in species evolution and lifestyle adaptation". BMC Genomics *13*(1): 533.

Canani, R. B., Costanzo, M. Di, Leone, L., Pedata, M., Meli, R., & Calignano, A. (2011). "Potential beneficial effects of butyrate in intestinal and extraintestinal diseases". World Journal of Gastroenterology *17*(12): 1519–1528.

Couto, J., St-Louis, R., & Karboune, S. (2011). "Optimization of feruloyl esterase-catalyzed synthesis of feruloylated oligosaccharides by response surface methodology". Journal of Molecular Catalysis B: Enzymatic *73*(1–4): 53–62.

Di, R., Vakkalanka, M. S., Onumpai, C., Chau, H. K., White, A., Rastall, R. A., … Hotchkiss, A. T. (2017). "Pectic oligosaccharide structure-function relationships: Prebiotics, inhibitors of Escherichia coli O157:H7 adhesion and reduction of Shiga toxin cytotoxicity in HT29 cells". Food Chemistry *227*: 245–254.

Dongowski, G., & Lorenz, A. (1998). "Unsaturated oligogalacturonic acids are generated by in vitro treatment of pectin with human faecal flora". Carbohydrate Research *314*(3–4): 237–244.
Faulds, C. B., & Williamson, G. (1999). "The role of hydroxycinnamates in the plant cell wall". Journal of the Science of Food and Agriculture *79*(3): 393–395.
Giuliani, S., Piana, C., Setti, L., Hochkoeppler, A., Pifferi, P. G., Williamson, G., & Faulds, C. B. (2001). "Synthesis of pentylferulate by a feruloyl esterase from Aspergillus niger using water-in-oil microemulsions". Biotechnology Letters *23*(4): 325–330.
Gómez, B., Gullón, B., Remoroza, C., Schols, H. A., Parajó, J. C., & Alonso, J. L. (2014). "Purification, characterization, and prebiotic properties of pectic oligosaccharides from orange peel wastes.". Journal of Agricultural and Food Chemistry *62*(40): 9769–82.
Gong, L., Wang, H., Wang, T., Liu, Y., Wang, J., & Sun, B. (2019). "Feruloylated oligosaccharides modulate the gut microbiota in vitro via the combined actions of oligosaccharides and ferulic acid". Journal of Functional Foods *60*: 103453.
Graf, E. (1992). "Antioxidant potential of ferulic acid". Free Radical Biology and Medicine *13*(4): 435–448.
Holck, J., Lorentzen, A., Vigsnæs, L. K., Licht, T. R., Mikkelsen, J. D., & Meyer, A. S. (2011). "Feruloylated and nonferuloylated arabino-oligosaccharides from sugar beet pectin selectively stimulate the growth of Bifidobacterium spp. in human fecal in vitro fermentations.". Journal of Agricultural and Food Chemistry *59*(12): 6511–9.
Khodaei, N., & Karboune, S. (2013). "Extraction and structural characterisation of rhamnogalacturonan I-type pectic polysaccharides from potato cell wall". Food Chemistry *139*(1–4): 617–623.
Kim, J.-H., Block, D. E., Shoemaker, S. P., & Mills, D. A. (2010). "Atypical ethanol production by carbon catabolite derepressed lactobacilli". Bioresource Technology *101*(22): 8790–8797.
Lin, B., Gong, J., Wang, Q., Cui, S., Yu, H., & Huang, B. (2011). "In-vitro assessment of the effects of dietary fibers on microbial fermentation and communities from large intestinal digesta of pigs". Food Hydrocolloids *25*(2): 180–188.
Mäkelä, M. R., Marinović, M., Nousiainen, P., Liwanag, A. J. M., Benoit, I., Sipilä, J., … Hildén, K. S. (2015). "Aromatic Metabolism of Filamentous Fungi in Relation to the Presence of Aromatic Compounds in Plant Biomass". In *Advances in Applied Microbiology* (Vol. 91, pp. 63–137). Academic Press.
Mastihubová, M., Mastihuba, V., Bilaničová, D., & Boreková, M. (2006). "Commercial enzyme preparations catalyse feruloylation of glycosides". Journal of Molecular Catalysis B: Enzymatic *38*(1): 54–57.
NCBI Resource Coordinators. (2018). "Database resources of the National Center for Biotechnology Information". Nucleic Acids Research *46*(D1): D8–D13.
Oosterveld, A., Beldman, G., Schols, H. A., & Voragen, A. G. J. (2000). "Characterization of arabinose and ferulic acid rich pectic polysaccharides and hemicelluloses from sugar beet pulp". Carbohydrate Research *328*(2): 185–197.
Ou, J., & Sun, Z. (2014). "Feruloylated oligosaccharides: Structure, metabolism and function". Journal of Functional Foods *7*: 90–100.
Ralet, M.-C., Thibault, J.-F., Faulds, C. B., & Williamson, G. (1994). "Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp". Carbohydrate Research *263*(2): 227–241.
Rohn, S., Rawel, H. M., & Kroll, J. (2002). "Inhibitory Effects of Plant Phenols on the Activity of Selected Enzymes". Journal of Agricultural and Food Chemistry *50*(12): 3566–3571.

Rudjito, R. C., Ruthes, A. C., Jiménez-Quero, A., & Vilaplana, F. (2019). "Feruloylated Arabinoxylans from Wheat Bran: Optimization of Extraction Process and Validation at Pilot Scale". ACS Sustainable Chemistry & Engineering *7*(15): 13167–13177.

Ruiz-Garcia, Y., Smith, P. A., & Bindon, K. A. (2014). "Selective extraction of polysaccharide affects the adsorption of proanthocyanidin by grape cell walls". Carbohydrate Polymers *114*: 102–114.

Sato, N., Takano, Y., Mizuno, M., Nozaki, K., Umemura, S., Matsuzawa, T., … Makishima, S. (2013). "Production of feruloylated arabino-oligosaccharides (FA-AOs) from beet fiber by hydrothermal treatment". The Journal of Supercritical Fluids *79*: 84–91.

Shoaib, M., Shehzad, A., Omar, M., Rakha, A., Raza, H., Sharif, H. R., … Niazi, S. (2016). "Inulin: Properties, health benefits and food applications". Carbohydrate Polymers *147*: 444–454.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). "[14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent". In *Methods in Enzymology* (Vol. 299, pp. 152–178). Academic Press.

Szwajgier, D., & Jakubczyk, A. (2010). "Biotransformation of ferulic acid by Lactobacillus acidophilus K1 and selected Bifidobacterium strains". Acta Scientiarum Polonorum, Technologia Alimentaria *9*: 45–59.

Tamayo-Cabezas, J., & Karboune, S. (2019). "Immobilized feruloyl esterase from Humicola insolens catalyzes the synthesis of feruloylated oligosaccharides". Process Biochemistry *79*: 81–90.

Thörn, C., Gustafsson, H., & Olsson, L. (2011). "Immobilization of feruloyl esterases in mesoporous materials leads to improved transesterification yield". Journal of Molecular Catalysis B: Enzymatic *72*(1–2): 57–64.

Tingirikari, J. M. R. (2019). "In-Vitro Prebiotic Analysis of Microbiota Accessible Pectic Polysaccharides". Current Microbiology *76*(12): 1452–1460.

Topakas, E., Stamatis, H., Biely, P., Kekos, D., Macris, B. ., & Christakopoulos, P. (2003). "Purification and characterization of a feruloyl esterase from Fusarium oxysporum catalyzing esterification of phenolic acids in ternary water–organic solvent mixtures". Journal of Biotechnology *102*(1): 33–44.

Turquois, T., Rinaudo, M., Taravel, F. ., & Heyraud, A. (1999). "Extraction of highly gelling pectic substances from sugar beet pulp and potato pulp: influence of extrinsic parameters on their gelling properties". Food Hydrocolloids *13*(3): 255–262.

Vafiadi, C., Topakas, E., & Christakopoulos, P. (2006). "Regioselective esterase-catalyzed feruloylation of L-arabinobiose.". Carbohydrate Research *341*(12): 1992–1997.

Yang, L., Dordick, J. S., & Garde, S. (2004). "Hydration of Enzyme in Nonaqueous Media Is Consistent with Solvent Dependence of Its Activity". Biophysical Journal *87*(2): 812–821.

Yuan, X., Wang, J., Yao, H., & Chen, F. (2005). "Free radical-scavenging capacity and inhibitory activity on rat erythrocyte hemolysis of feruloyl oligosaccharides from wheat bran insoluble dietary fiber". LWT - Food Science and Technology *38*(8): 877–883.

Zhang, W., Fan, X., Gu, X., Gong, S., Wu, J., Wang, Z., … Wang, S. (2020). "Emulsifying properties of pectic polysaccharides obtained by sequential extraction from black tomato pomace". Food Hydrocolloids *100*: 105454.

Zhao, W., Chen, H., Wu, L., Ma, W., & Xie, Y. (2018). "Antioxidant properties of feruloylated oligosaccharides of different degrees of polymerization from wheat bran". Glycoconjugate Journal *35*(6): 547–559.

CHAPTER VII. GENERAL SUMMARY AND CONCLUSIONS

This research was centered on the extraction and characterization of cranberry cell wall polysaccharides and their derivatives that can be obtained by their hydrolysis (oligosaccharides) and esterification (ferulic acid esters). Plant cell wall is a very abundant biological material, and considerable efforts towards optimizing its utilization as source of energy and value-added chemicals are being made. However, cell wall is characterized by the presence of a matrix of different polymers that varies significantly in composition and distribution of intermolecular bonds across species and tissues, and most of the research has focused on lignified and cellulose-rich cell walls, such as those typically found in trunks and stalks.

The cell walls of fruits, on the other hand, contain very little lignin, and are very rich in polysaccharides, of which cellulose represents only a fraction. While less abundant then wood in absolute terms, the fruit processing industry produces large quantities of by-products enriched in fruit cell wall material, and the development of approaches for its valorization is important to reduce waste. Several studies have in fact analyzed the cell wall composition of a variety of fruits, but cranberry has until now been primarily investigated for its polyphenolic content, leaving the much more abundant polysaccharide component scarcely characterized.

A sequential extraction approach with aqueous solvents under increasingly harsh conditions was applied to cranberry cell wall material. This approach will extract the polysaccharides from plant cell wall based on the type of interactions that stabilized them in the matrix. Hot acetate buffer extract was found to contain mostly pectic polysaccharides and smaller amounts of hemicellulose. Pectic polysaccharides in this extract were characterized by neutral branches with abundant arabinan chains, while galactose appeared to be included as short, branched arabinogalactan backbones of both types. Chelating agents extract had the highest content of galacturonic acid and the least hairy regions, indicating the prevalence of homogalacturonan. The composition of the neutral branches was analogous to the one in the first extract. Diluted alkali was also rich in pectic polysaccharides, with a reduced content of hemicelluloses and the highest abundance of neutral branches that, while rich in arabinan, were found to contain longer galactan chains than the other extracts. Finally, concentrate alkali extract contained mostly hemicelluloses, associated with arabinan from the ‘hairy’ regions of pectic polysaccharides. As all extracts contained both pectic and hemicellulosic structures, the two classes of polysaccharides appeared tightly connected in the cell wall material. A more detailed analysis of the structural properties of pectic polysaccharides was performed, in the face of recent developments presenting this class of molecules as valuable for prebiotic effect and the generation of beneficial oligosaccharides.

Chelating agents extract and diluted alkali extract, richest in pectic polysaccharides, were fractioned by anion exchange and gel filtration, obtaining populations of pectic polysaccharides with uniform charge density and molecular weight. Pure pectin-degrading enzymatic activities were employed to fragment the purified polysaccharides, and the analysis of the fragments made it possible to reconstruct several of their structural features, such as the percentage of galacturonic acid residues that constitute homogalacturonan, the presence and distribution of methyl esterification, and the abundance and composition of the neutral branches. The analyzed fractions resulted in overall poor in neutral branches, that appeared to be mainly composed of type I arabinogalactan with short galactan backbone heavily branched with arabinose. Hemicellulose residues appeared to be covalently bound to these structures. Linear galactan was confirmed to be a side chain in some of the pectic polysaccharides of diluted alkali extract. The presence of galactoarabinan, a more uncommon type of neutral branch, was strongly suggested by the observations.

Extraction of oligosaccharides was achieved from cranberry cell wall material by microwave-assisted alkaline extraction and by hydrolysis with commercial enzymatic and multi-enzymatic catalysts. While all tested microwave extraction conditions liberated compounds with degree of polymerization between 7 and 10, enriched in pectic oligosaccharides compared to the starting material, the enzymes released smaller fragments enriched in glucose. Three multi-enzymatic catalysts also generated extracts containing pectic neutral branches proportions in comparable to the microwave process. All multi-enzymatic catalysts had yields of extraction comparable or higher to that of microwave.

Feruloylation of chelating agents and diluted alkali extracts was achieved by the esterifying activity of *Humicola insolens* feruloyl esterase, immobilized on iminodiacetic acid-copper modified mesoporous epoxy-polyacrylic resin, using a surfactantless microemulsion of n-hexane, butanone and water 51:46:3 (%v/v). Extracts with and without feruloylation were compared as carbon sources for the anaerobic growth of selected strains of beneficial gut bacteria, finding that native extract promote growth as strongly or better than inulin, but feruloylation, specially of diluted alkali extract, has a negative impact.

Overall, this research contributes to the scientific knowledge providing a detailed description of the abundance and structural features of cranberry cell wall polysaccharides, with focus on pectic substances. It also offers a look at the feasibility of the generation from cranberry cell wall of oligosaccharides and modified polysaccharides, which may broaden the spectrum of applications for a material that is often considered only for its phenolic content.

CHAPTER VIII. CONTRIBUTIONS TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE STUDIES

8.1. Contributions to Knowledge

The major contributions to knowledge of this study are:

1. This is the first study to isolate and characterize the properties of oligosaccharides generated by microwave-assisted alkaline extraction from cranberry. The approach was found to be a rapid and simple way to obtain an extract enriched in pectic oligosaccharides that may present health promoting properties.

2. For the first time a sequential approach for the extraction of polysaccharide populations with different aqueous solvents has been applied to cranberry cell wall material determining yield, composition and glycosidic linkages of the polysaccharide thus obtained. This includes the first application of fragmentation with pure enzymatic activities as an analytical tool for the resolution of glycosidic linkages in cranberry polysaccharides. Detailed knowledge of the properties of cell wall polysaccharides facilitates the design of processes for the isolation of specific structures of interest as high value-added chemicals.

3. For the first time, the enzymatic esterification of polysaccharide extracts with ferulic acid was achieved by pure ferulic acid esterase in selected surfactantless microemulsions, obtaining polysaccharides with a significantly higher phenolic content. The effect of enzyme immobilization on iminodiacetic acid-copper modified epoxy-polyacrylic supports was assessed, finding it greatly enhances the enzyme's specific esterification activity.

4. This is the first study to assess the anaerobic fermentability of synthesized feruloylated polysaccharides by gut bacteria. Understanding the effect of polysaccharide modification on their prebiotic activity is important for evaluating their possible use as functional ingredients.

8.2. Recommendations for Future Research

- Confirmation of the presence of galactoarabinan branches in cranberry pectic polysaccharides by analysis of the enzymatically generated fragments by mass spectrometry with soft ionization techniques and/or nuclear magnetic resonance.

- Determination of the feruloylation pattern of the polysaccharides modified by *Humicola insolens* feruloyl esterase, specifically to determine whether the enzyme can catalyze esterification with ferulic acid's hydroxyl group as the nucleophile and the carboxylic group of galacturonic acid as the electrophile.

- More detailed analysis of the prebiotic activity of the obtained polysaccharides, oligosaccharides and feruloylated polysaccharides, by use of more strains of beneficial and commensal gut bacteria or fermentation by fecal inoculum.

REFERENCES

Aachary, A. A., & Prapulla, S. G. (2009). "Value addition to corncob: Production and characterization of xylooligosaccharides from alkali pretreated lignin-saccharide complex using Aspergillus oryzae MTCC 5154". Bioresource Technology *100*(2): 991–995.

Aachary, A. A., & Prapulla, S. G. (2011). "Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications". Comprehensive Reviews in Food Science and Food Safety *10*(1): 2–16.

Abid, M., Cheikhrouhou, S., Renard, C. M. G. C., Bureau, S., Cuvelier, G., Attia, H., & Ayadi, M. A. (2017). "Characterization of pectins extracted from pomegranate peel and their gelling properties". Food Chemistry *215*: 318–325.

Ablett, R. F. (2012, July 10). "Plant waste bio-product pomace extract concentrates and processes of producing same". (USA).

Akpinar, O., Ak, O., Kavas, A., Bakir, U., & Yilmaz, L. (2007). "Enzymatic Production of Xylooligosaccharides from Cotton Stalks". Journal of Agricultural and Food Chemistry *55*(14): 5544–5551.

Alboofetileh, M., Rezaei, M., Tabarsa, M., & You, S. (2019). "Ultrasound-assisted extraction of sulfated polysaccharide from Nizamuddinia zanardinii: Process optimization, structural characterization, and biological properties". Journal of Food Process Engineering *42*(2): e12979.

Allen, D. K., & Tao, B. Y. (1999). "Carbohydrate-alkyl ester derivatives as biosurfactants". Journal of Surfactants and Detergents *2*(3): 383–390.

Arzani, K., Khoshghalb, H., Malakouti, M. J., & Barzegar, M. (2011). "Total Oxalate-Soluble Pectin Concentration in Asian Pear (Pyrus serotina Rehd) Fruit in Relation to Ripening, Storage and Internal Browning Disorder". Journal of Agricultural Science and Technology *13*(4): 611–626.

Auerbach, M. H. ., Mitchell, H., & Moppett, F. K. . (2012). "Polydextrose". In *Alternative Sweeteners, Fourth Edition* (pp. 489–506).

Auh, J. H., Lee, H. . G., Kim, J. W., Kim, J. C., Yoon, H. S., & Park, K. H. (1999). "Highly Concentrated Branched Oligosaccharides as Cryoprotectant for Surimi". Journal of Food Science *64*(3): 418–422.

Axelos, M. A. V., Thibault, J. F., & Lefebvre, J. (1989). "Structure of citrus pectins and viscometric study of their solution properties". International Journal of Biological Macromolecules *11*(3): 186–191.

Baker, G. L., & Kneeland, R. F. (1936). "Cranberry Pectin Properties". Industrial & Engineering Chemistry *28*(3): 372–375.

Bar-Peled, M., Urbanowicz, B. R., & O'Neill, M. A. (2012). "The Synthesis and Origin of the Pectic Polysaccharide Rhamnogalacturonan II – Insights from Nucleotide Sugar Formation and Diversity". Frontiers in Plant Science *3*: 92.

Biwer, A., Antranikian, G., & Heinzle, E. (2002). "Enzymatic production of cyclodextrins.". Applied Microbiology and Biotechnology *59*(6): 609–17.

Blaker, K. M., & Olmstead, J. W. (2014). "Stone Cell Frequency and Cell Area Variation of Crisp and Standard Texture Southern Highbush Blueberry Fruit". J. Amer. Soc. Hort. Sci. *139*(5): 553–557.

Bonnin, E., Garnier, C., & Ralet, M.-C. (2014). "Pectin-modifying enzymes and pectin-derived materials: applications and impacts.". Applied Microbiology and Biotechnology *98*(2): 519–532.

Brett, C., Baydoun, E.-H., & Abdel-Massih, R. (2005). "Pectin–xyloglucan linkages in type I primary cell walls of plants". Plant Biosystems - An International Journal Dealing with All Aspects of Plant Biology *139*(1): 54–59.

Brownlee, I. A. (2011). "The physiological roles of dietary fibre". Food Hydrocolloids *25*(2): 238–250.

Brummel, S. E., & Lee, K. (1990). "Soluble Hydrocolloids Enable Fat Reduction in Process Cheese Spreads". Journal of Food Science *55*(5): 1290–1292.
Buchanan, B. B., Gruissem, W., Jones, R. L., & Vickers, K. (Eds.). (2015). *Biochemistry & molecular biology of plants* (2nd ed.). John Wiley & Sons, Incorporated.
Burcelin, R., Courtney, M., & Amar, J. (2015). "Gut Microbiota and Metabolic Diseases: From Pathogenesis to Therapeutic Perspective". In S. Kochhar & F.-P. Martin (Eds.), *Metabonomics and Gut Microbiota in Nutrition and Disease* (pp. 199–234). Springer London (London).
Burger, O., Weiss, E., Sharon, N., Tabak, M., Neeman, I., & Ofek, I. (2002). "Inhibition of *Helicobacter pylori* Adhesion to Human Gastric Mucus by a High-Molecular-Weight Constituent of Cranberry Juice". Critical Reviews in Food Science and Nutrition *42*(sup3): 279–284.
Burton, R. A., & Fincher, G. B. (2009). "(1,3;1,4)-β-D-Glucans in Cell Walls of the Poaceae, Lower Plants, and Fungi: A Tale of Two Linkages". Molecular Plant *2*(5): 873–882.
Chandra, K., Ghosh, K., Ojha, A. K., & Islam, S. S. (2009). "Chemical analysis of a polysaccharide of unripe (green) tomato (Lycopersicon esculentum)". Carbohydrate Research *344*(16): 2188–2194.
Chen, J., Liang, R., Liu, W., Li, T., Liu, C., Wu, S., & Wang, Z. (2013). "Pectic-oligosaccharides prepared by dynamic high-pressure microfluidization and their in vitro fermentation properties.". Carbohydrate Polymers *91*(1): 175–82.
Chen, Y., Luo, H., Gao, A., & Zhu, M. (2011). "Ultrasound-assisted extraction of polysaccharides from litchi (Litchi chinensis Sonn.) seed by response surface methodology and their structural characteristics". Innovative Food Science & Emerging Technologies *12*(3): 305–309.
Clydesdale, F. M., Main, J. H., & Francis, F. J. (1979). "Cranberry Pigments as Colorants for Beverages and Gelatin Desserts". Journal of Food Protection *3*: 196–276.
Coenen, G. J., Bakx, E. J., Verhoef, R. P., Schols, H. A., & Voragen, A. G. J. (2007). "Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan type I". Carbohydrate Polymers *70*(2): 224–235.
Coimbra, M. A., Waldron, K. W., & Selvendran, R. R. (1994). "Isolation and characterisation of cell wall polymers from olive pulp (Olea europaea L.)". Carbohydrate Research *252*: 245–262.
Colquhoun, I. J., Ralet, M.-C., Thibault, J.-F., Faulds, C. B., & Williamson, G. (1994). "Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy". Carbohydrate Research *263*(2): 243–256.
Combo, A. M. M., Aguedo, M., Quiévy, N., Danthine, S., Goffin, D., Jacquet, N., … Paquot, M. (2013). "Characterization of sugar beet pectic-derived oligosaccharides obtained by enzymatic hydrolysis.". International Journal of Biological Macromolecules *52*: 148–56.
Contreras-Esquivel, J. C., Voget, C. E., Vita, C. E., Perez, E. J. D., & Renard, C. M. G. C. (2006). "Enzymatic Extraction of Lemon Pectin by Endo-Polygalacturonase from Aspergillus niger". Food Science and Biotechnology *15*(2): 163–167.
Coulier, L., Zha, Y., Bas, R., & Punt, P. J. (2013). "Analysis of oligosaccharides in lignocellulosic biomass hydrolysates by high-performance anion-exchange chromatography coupled with mass spectrometry (HPAEC–MS)". Bioresource Technology *133*: 221–231.
Crittenden, R. G., & Playne, M. J. (1996). "Production, properties and applications of food-grade oligosaccharides". Trends in Food Science & Technology *7*(11): 353–361.
Cutillas-Iturralde, A., Zarra, I., Fry, S. C., & Lorences, E. P. (1994). "Implication of persimmon fruit hemicellulose metabolism in the softening process. Importance of xyloglucan endotransglycosylase". Physiologia Plantarum *91*(2): 169–176.
Davis, F., & Higson, S. (2011). "Cyclodextrins". In *Macrocycles: Construction, Chemistry and Nanotechnology Applications* (pp. 190–254). John Wiley & Sons, Ltd.

Deery, M. J., Stimson, E., & Chappell, C. G. (2001). "Size exclusion chromatography/mass spectrometry applied to the analysis of polysaccharides.". Rapid Communications in Mass Spectrometry : RCM *15*(23): 2273–83.

Devulapalle, K. S., Gómez de Segura, A., Ferrer, M., Alcalde, M., Mooser, G., & Plou, F. J. (2004). "Effect of carbohydrate fatty acid esters on Streptococcus sobrinus and glucosyltransferase activity.". Carbohydrate Research *339*(6): 1029–34.

Dickinson, E. (2009). "Hydrocolloids as emulsifiers and emulsion stabilizers". Food Hydrocolloids *23*(6): 1473–1482.

Du, B., Song, Y., Hu, X., Liao, X., Ni, Y., & Li, Q. (2011). "Oligosaccharides prepared by acid hydrolysis of polysaccharides from pumpkin (Cucurbita moschata) pulp and their prebiotic activities". International Journal of Food Science & Technology *46*(5): 982–987.

Duus, J. Ø., Gotfredsen, C. H., & Bock, K. (2000). "Carbohydrate Structural Determination by NMR Spectroscopy: Modern Methods and Limitations †". Chemical Reviews *100*(12): 4589–4614.

Ebringerová, A., & Hromádková, Z. (2010). "An overview on the application of ultrasound in extraction, separation and purification of plant polysaccharides". Open Chemistry *8*(2): 243–257.

Evert, R. F. (2006). *Esau's Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function, and Development*. John Wiley & Sons.

Fang, H.-Y., Chen, Y.-K., Chen, H.-H., Lin, S.-Y., & Fang, Y.-T. (2012). "Immunomodulatory effects of feruloylated oligosaccharides from rice bran". Food Chemistry. *134*(2): 836—840.

Fishman, M. L., Chau, H. K., Hoagland, P., & Ayyad, K. (1999). "Characterization of pectin, flash-extracted from orange albedo by microwave heating, under pressure". Carbohydrate Research *323*(1–4): 126–138.

Fry, S. C. (2010). *Annual Plant Reviews*. (P. Ulvskov, Ed.)*Annual Plant Reviews Volume 41* (Vol. 41). Wiley-Blackwell (Oxford, UK).

Fry, S. C., York, W. S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., … White, A. R. (1993). "An unambiguous nomenclature for xyloglucan-derived oligosaccharides". Physiologia Plantarum *89*(1): 1–3.

Fujio, Y., & Furuta, S. (1989). "Fractionation of water insoluble solids and extraction of pectins from residues of juice-extracted satsuma mandarin fruit by wet grinding". International Journal of Food Science & Technology *24*(4): 439–445.

Funakawa, H., & Miwa, K. (2015). "Synthesis of borate cross-linked rhamnogalacturonan II". Frontiers in Plant Science *6*: 223.

Galermo, A. G., Nandita, E., Barboza, M., Amicucci, M. J., Vo, T.-T. T., & Lebrilla, C. B. (2018). "Liquid Chromatography–Tandem Mass Spectrometry Approach for Determining Glycosidic Linkages". Analytical Chemistry *90*(21): 13073–13080.

Ganan, M., Collins, M., Rastall, R., Hotchkiss, A. T., Chau, H. K., Carrascosa, A. V., & Martinez-Rodriguez, A. J. (2010). "Inhibition by pectic oligosaccharides of the invasion of undifferentiated and differentiated Caco-2 cells by Campylobacter jejuni". International Journal of Food Microbiology *137*(2–3): 181–185.

Garozzo, D., Spina, E., Cozzolino, R., Cescutti, P., & Fett, W. F. (1999). "Studies on the primary structure of short polysaccharides using SEC MALDI mass spectroscopy". Carbohydrate Research *323*(1–4): 139–146.

Giusti, M. M., & Wallace, T. C. (2009). "Flavonoids as Natural Pigments". In T. Bechtold & R. Mussak (Eds.), *Handbook of Natural Colorants* (pp. 257–275). John Wiley & Sons, Ltd (Chippenham, GB).

Gómez, B., Gullón, B., Remoroza, C., Schols, H. A., Parajó, J. C., & Alonso, J. L. (2014). "Purification, characterization, and prebiotic properties of pectic oligosaccharides from orange peel wastes.".

Journal of Agricultural and Food Chemistry *62*(40): 9769–82.
Gong, L., Wang, H., Wang, T., Liu, Y., Wang, J., & Sun, B. (2019). "Feruloylated oligosaccharides modulate the gut microbiota in vitro via the combined actions of oligosaccharides and ferulic acid". Journal of Functional Foods *60*: 103453.
Grasdalen, H., Einar Bakøy, O., & Larsen, B. (1988). "Determination of the degree of esterification and the distribution of methylated and free carboxyl groups in pectins by 1H-n.m.r. spectroscopy". Carbohydrate Research *184*: 183–191.
Gray, J. A., & BeMiller, J. N. (2003). "Bread Staling: Molecular Basis and Control". Comprehensive Reviews in Food Science and Food Safety *2*(1): 1–21.
Greffe, L., Bessueille, L., Bulone, V., & Brumer, H. (2005). "Synthesis , preliminary characterization , and application of novel surfactants from highly branched xyloglucan oligosaccharides". Glycobiology *15*(4): 437–445.
Grigelmo-Miguel, N., & Martín-Belloso, O. (1999). "Influence of fruit dietary fibre addition on physical and sensorial properties of strawberry jams". Journal of Food Engineering *41*(1): 13–21.
Gruber, L., & Haller, D. (2015). "Role of the Gut Microbiota in Maintaining GI Health: Highlights on Inflammatory Bowel Disease". In S. Kochhar & F.-P. Martin (Eds.), *Metabonomics and Gut Microbiota in Nutrition and Disease* (pp. 261–310). Springer London (London).
Harris, P. J., & Smith, B. G. (2006). "Plant cell walls and cell-wall polysaccharides: structures, properties and uses in food products". International Journal of Food Science and Technology *41*(s2): 129–143.
Harrison, J. E., Oomah, B. D., Diarra, M. S., & Ibarra-Alvarado, C. (2013). "Bioactivities of pilot-scale extracted cranberry juice and pomace". Journal of Food Processing and Preservation *37*(4): 356–365.
Hass, H. B. (1968). "Early history of sucrose esters". In *Sugar esters* (pp. 1–7). Noyes Development Corp. (Park Ridge N.J.).
Heredia, A., Jiménez, A., & Guillén, R. (1995). "Composition of plant cell walls". Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung *200*(1): 24–31.
Hilz, H., Bakx, E. J., Schols, H. A., & Voragen, A. G. J. (2005). "Cell wall polysaccharides in black currants and bilberries—characterisation in berries, juice, and press cake". Carbohydrate Polymers *59*(4): 477–488.
Hilz, H., de Jong, L. E., Kabel, M. A., Verhoef, R., Schols, H. A., & Voragen, A. G. J. (2007). "Bilberry xyloglucan--novel building blocks containing beta-xylose within a complex structure.". Carbohydrate Research *342*(2): 170–81.
Hilz, H., Williams, P., Doco, T., Schols, H. A., & Voragen, A. G. J. (2006). "The pectic polysaccharide rhamnogalacturonan II is present as a dimer in pectic populations of bilberries and black currants in muro and in juice". Carbohydrate Polymers *65*(4): 521–528.
Holck, J., Hjernø, K., Lorentzen, A., Vigsnæs, L. K., Hemmingsen, L., Licht, T. R., … Meyer, A. S. (2011). "Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after in vitro fermentation". Process Biochemistry *46*(5): 1039–1049.
Holck, J., Hotchkiss, A. T., Meyer, A. S., Mikkelsen, J. D., & Rastall, R. A. (2014). "Production and Bioactivity of Pectic Oligosaccharides from Fruit and Vegetable Biomass". In F. J. Moreno & M. L. Sanz (Eds.), *Food Oligosaccharides* (pp. 76–87). John Wiley & Sons, Ltd (Chichester, UK).
Holck, J., Lorentzen, A., Vigsnæs, L. K., Licht, T. R., Mikkelsen, J. D., & Meyer, A. S. (2011). "Feruloylated and nonferuloylated arabino-oligosaccharides from sugar beet pectin selectively stimulate the growth of Bifidobacterium spp. in human fecal in vitro fermentations.". Journal of Agricultural and Food Chemistry *59*(12): 6511–9.

Holmes, A. B., & Rha, C. (1978). "Structure and chemical composition of cranberry cell wall material". Journal of Food Science *43*(1): 112–120.
Howard, M. D., Gordon, D. T., Garleb, K. A., & Kerley, M. S. (1995). "Dietary Fructooligosaccharide, Xylooligosaccharide and Gum Arabic Have Variable Effects on Cecal and Colonic Microbiota and Epithelial Cell Proliferation in Mice and Rats". The Journal of Nutrition *125*(10): 2604–2609.
Howell, A. B., Reed, J. D., Krueger, C. G., Winterbottom, R., Cunningham, D. G., & Leahy, M. (2005). "A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity.". Phytochemistry *66*(18): 2281–91.
Hu, F. B., Manson, J. E., Stampfer, M. J., Colditz, G., Liu, S., Solomon, C. G., & Willett, W. C. (2001). "Diet, Lifestyle, and the Risk of Type 2 Diabetes Mellitus in Women". New England Journal of Medicine *345*(11): 790–797.
Imeson, A. (Ed.). (1992). *Thickening and Gelling Agents for Food*. Springer US (Boston, MA).
Jayapal, N., Samanta, A. K., Kolte, A. P., Senani, S., Sridhar, M., Suresh, K. P., & Sampath, K. T. (2013). "Value addition to sugarcane bagasse: Xylan extraction and its process optimization for xylooligosaccharides production". Industrial Crops and Products *42*: 14–24.
Jeong, H.-S., Kim, H.-Y., Ahn, S. H., Oh, S. C., Yang, I., & Choi, I.-G. (2014). "Optimization of enzymatic hydrolysis conditions for extraction of pectin from rapeseed cake (Brassica napus L.) using commercial enzymes". Food Chemistry *157*: 332–338.
Ji, L., Jie, Z., Ying, X., Yue, Q., Zhou, Y., & Sun, L. (2018). "Structural characterization of alkali-soluble polysaccharides from Panax ginseng C. A. Meyer". Royal Society Open Science *5*(3): 171644.
Joye, D. ., & Luzio, G. . (2000). "Process for selective extraction of pectins from plant material by differential pH". Carbohydrate Polymers *43*(4): 337–342.
Khan, M., Bibi, N., & Zeb, A. (2015). "Optimization of Process Conditions for Pectin Extraction from Citrus Peel". Science, Technology and Development *34*(1): 9–15.
Khodaei, N., & Karboune, S. (2013). "Extraction and structural characterisation of rhamnogalacturonan I-type pectic polysaccharides from potato cell wall". Food Chemistry *139*(1–4): 617–623.
Khodaei, N., Karboune, S., & Orsat, V. (2016). "Microwave-assisted alkaline extraction of galactan-rich rhamnogalacturonan I from potato cell wall by-product". Food Chemistry *190*: 495–505.
Kikuchi, A., Edashige, Y., Ishii, T., & Satoh, S. (1996). "A xylogalacturonan whose level is dependent on the size of cell clusters is present in the pectin from cultured carrot cells". Planta *200*(4): 369–372.
Kim, S., Kim, W., & Hwang, I. K. (2003). "Optimization of the extraction and purification of oligosaccharides from defatted soybean meal". International Journal of Food Science and Technology *38*(3): 337–342.
Kobayashi, M., Matoh, T., & Azuma, J. (1996). "Two Chains of Rhamnogalacturonan II Are Cross-Linked by Borate-Diol Ester Bonds in Higher Plant Cell Walls". Plant Physiology *110*(3): 1017–1020.
Kohmoto, T., Fukui, F., Takaku, H., & Mitsuoka, T. (1991). "Dose-response Test of Isomaltooligosaccharides for Increasing Fecal Bifidobacteria.". Agricultural and Biological Chemistry *55*(8): 2157–2159.
Kolanowski, W., Laufenberg, G., & Kunz, B. (2004). "Fish oil stabilisation by microencapsulation with modified cellulose.". International Journal of Food Sciences and Nutrition *55*(4): 333–43.
Kumar, J. K., & Sinha, A. K. (2004). "Resurgence of natural colourants: a holistic view.". Natural Product Research *18*(1): 59–84.
Lahaye, M., Quemener, B., Causse, M., & Seymour, G. B. (2012). "Hemicellulose fine structure is affected differently during ripening of tomato lines with contrasted texture". International Journal

of Biological Macromolecules *51*(4): 462–470.
Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., & Fernández-Bolaños, J. (2012). "Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated". Food Hydrocolloids *28*(1): 92–104.
Lee, S. H., Ha, S. H., Dang, D. T., Chang, W.-J., & Koo, Y.-M. (2007). "Lipase-catalyzed synthesis of fatty acid sugar ester using supersaturated sugar solution in ionic liquids". Journal of Biotechnology *131*(2): S88.
Levy, S., & Staehelin, L. A. (1992). "Synthesis, assembly and function of plant cell wall macromolecules". Current Opinion in Cell Biology *4*(5): 856–862.
Li, B., Dobruchowska, J. M., Gerwig, G. J., Dijkhuizen, L., & Kamerling, J. P. (2013). "Structural investigation of water-soluble polysaccharides extracted from the fruit bodies of Coprinus comatus.". Carbohydrate Polymers *91*(1): 314–21.
Li, P., Xia, J., Nie, Z., & Shan, Y. (2016). "Pectic oligosaccharides hydrolyzed from orange peel by fungal multi-enzyme complexes and their prebiotic and antibacterial potentials". LWT - Food Science and Technology *69*: 203–210.
Li, S., Li, T., Zhu, R., Wang, N., Song, Y., Wang, S., & Guo, M. (2013). "Antibacterial Action of Haw Pectic Oligosaccharides". International Journal of Food Properties *16*(3): 706–712.
Lim, J., Yoo, J., Ko, S., & Lee, S. (2012). "Extraction and characterization of pectin from Yuza (Citrus junos) pomace: A comparison of conventional-chemical and combined physical–enzymatic extractions". Food Hydrocolloids *29*(1): 160–165.
Liu, L., Cao, J., Huang, J., Cai, Y., & Yao, J. (2010). "Extraction of pectins with different degrees of esterification from mulberry branch bark". Bioresource Technology *101*(9): 3268–3273.
Locke, A. B. (2018). "Urinary Tract Infection (UTI)". In *Integrative Medicine* (pp. 211-217.e2). Elsevier.
Macpherson, A. J., & Harris, N. L. (2004). "Opinion: Interactions between commensal intestinal bacteria and the immune system". Nature Reviews. Immunology *4*(6): 478–485.
Manderson, K., Pinart, M., Tuohy, K. M., Grace, W. E., Hotchkiss, A. T., Widmer, W., … Rastall, R. A. (2005). "In Vitro Determination of Prebiotic Properties of Oligosaccharides Derived from an Orange Juice Manufacturing By-Product Stream". Applied and Environmental Microbiology *71*(12): 8383–8389.
Marlett, J., & Vollendorf, N. (1994). "Dietary fiber content and composition of different forms of fruits". Food Chemistry *51*(1): 39–44.
Martínez Sabajanes, M., Yáñez, R., Alonso, J. L., & Parajó, J. C. (2012). "Pectic oligosaccharides production from orange peel waste by enzymatic hydrolysis". International Journal of Food Science & Technology *47*(4): 747–754.
Massiot, P. (1994). "Characterisation and enzymatic hydrolysis of cell-wall polysaccharides from different tissue zones of apple". Carbohydrate Polymers *25*(3): 145–154.
Mastihubová, M., Mastihuba, V., Bilaničová, D., & Boreková, M. (2006). "Commercial enzyme preparations catalyse feruloylation of glycosides". Journal of Molecular Catalysis B: Enzymatic *38*(1): 54–57.
Matora, A. V., Korshunova, V. E., Shkodina, O. G., Zhemerichkin, D. A., Ptitchkina, N. M., & Morris, E. R. (1995). "The application of bacterial enzymes for extraction of pectin from pumpkin and sugar beet". Food Hydrocolloids *9*(1): 43–46.
Mazumder, S., Lerouge, P., Loutelierbourhis, C., Driouich, A., & Ray, B. (2005). "Structural characterisation of hemicellulosic polysaccharides from using specific enzyme hydrolysis, ion exchange chromatography and MALDI-TOF mass spectroscopy". Carbohydrate Polymers *59*(2): 231–238.

Meents, M. J., Watanabe, Y., & Samuels, A. L. (2018). "The cell biology of secondary cell wall biosynthesis". Annals of Botany *121*(6): 1107–1125.
Mohnen, D. (2008). "Pectin structure and biosynthesis.". Current Opinion in Plant Biology *11*(3): 266–277.
Monro, J. A., Penny, D., & Bailey, R. W. (1976). "The organization and growth of primary cell walls of lupin hypocotyl". Phytochemistry *15*(8): 1193–1198.
Moon, J. S., Shin, S. Y., Choi, H. S., Joo, W., Cho, S. K., Li, L., … Han, N. S. (2015). "In vitro digestion and fermentation properties of linear sugar-beet arabinan and its oligosaccharides". Carbohydrate Polymers *131*: 50–56.
Mravec, J., Kračun, S. K., Rydahl, M. G., Westereng, B., Pontiggia, D., De Lorenzo, G., … Willats, W. G. T. (2017). "An oligogalacturonide-derived molecular probe demonstrates the dynamics of calcium-mediated pectin complexation in cell walls of tip-growing structures". The Plant Journal *91*(3): 534–546.
Müller-Maatsch, J., Caligiani, A., Tedeschi, T., Elst, K., & Sforza, S. (2014). "Simple and validated quantitative ^{1}H NMR method for the determination of methylation, acetylation, and feruloylation degree of pectin.". Journal of Agricultural and Food Chemistry *62*(37): 9081–7.
Muñoz-Almagro, N., Valadez-Carmona, L., Mendiola, J. A., Ibáñez, E., & Villamiel, M. (2019). "Structural characterisation of pectin obtained from cacao pod husk. Comparison of conventional and subcritical water extraction". Carbohydrate Polymers *217*: 69–78.
Nabarlatz, D., Farriol, X., & Montané, D. (2005). "Autohydrolysis of Almond Shells for the Production of Xylo-oligosaccharides: Product Characteristics and Reaction Kinetics". Industrial & Engineering Chemistry Research *44*(20): 7746–7755.
Nakakuki, T. (2003). "Development of Functional Oligosaccharides in Japan". Trends in Glycoscience and Glycotechnology *15*(82): 57–64.
Neto, C. C., & Vinson, J. A. (2011). "Cranberry". In I. F. F. Benzie & S. Wachtel-Galor (Eds.), *Herbal Medicine: Biomolecular and Clinical Aspects* (2nd ed.). CRC Press/Taylor & Francis (Boca Raton).
Ng, J. K. T., Zujovic, Z. D., Smith, B. G., Johnston, J. W., Schröder, R., & Melton, L. D. (2014). "Solid-state 13C NMR study of the mobility of polysaccharides in the cell walls of two apple cultivars of different firmness.". Carbohydrate Research *386*: 1–6.
Oechslin, R., Lutz, M. V, & Amadò, R. (2003). "Pectic substances isolated from apple cellulosic residue: structural characterisation of a new type of rhamnogalacturonan I". Carbohydrate Polymers *51*(3): 301–310.
Ohta, T., Yamasaki, S., Egashira, Y., & Sanada, H. (1994). "Antioxidative Activity of Corn Bran Hemicellulose Fragments". Journal of Agricultural and Food Chemistry *42*(3): 653–656.
Olano-Martin, E., Gibson, G. R., & Rastall, R. A. (2002). "Comparison of the in vitro bifidogenic properties of pectins and pectic-oligosaccharides". Journal of Applied Microbiology *93*(3): 505–511.
Oomen, R. J. F. J., Vincken, J.-P., Bush, M. S., Skjøt, M., Doeswijk-Voragen, C. H. L., Ulvskov, P., … Visser, R. G. F. (2003). *Advances in Pectin and Pectinase Research*. (F. Voragen, H. Schols, & R. Visser, Eds.)*Advances in Pectin and Pectinase Research*. Springer Netherlands (Dordrecht).
Ou, J., Carbonero, F., Zoetendal, E. G., DeLany, J. P., Wang, M., Newton, K., … O'Keefe, S. J. (2013). "Diet, microbiota, and microbial metabolites in colon cancer risk in rural Africans and African Americans". The American Journal of Clinical Nutrition *98*(1): 111–120.
Ou, S., Li, A., & Yang, A. (2001). "A study on synthesis of starch ferulate and its biological properties". Food Chemistry *74*(1): 91–95.

Paniagua, C., Posé, S., Morris, V. J., Kirby, A. R., Quesada, M. A., & Mercado, J. A. (2014). "Fruit softening and pectin disassembly: an overview of nanostructural pectin modifications assessed by atomic force microscopy". Annals of Botany *114*(6): 1375–1383.

Park, S.-I., & Zhao, Y. (2006). "Development and Characterization of Edible Films from Cranberry Pomace Extracts". Journal of Food Science *71*(2): E95–E101.

Patel, S., & Goyal, A. (2011). "Functional oligosaccharides: production, properties and applications". World Journal of Microbiology and Biotechnology *27*(5): 1119–1128.

Peña, M. J., & Carpita, N. C. (2004). "Loss of highly branched arabinans and debranching of rhamnogalacturonan I accompany loss of firm texture and cell separation during prolonged storage of apple.". Plant Physiology *135*(3): 1305–13.

Peng, Q., Lv, X., Xu, Q., Li, Y., Huang, L., & Du, Y. (2012). "Isolation and structural characterization of the polysaccharide LRGP1 from Lycium ruthenicum.". Carbohydrate Polymers *90*(1): 95–101.

Pérez-Victoria, I., & Morales, J. C. (2006). "Regioselectivity in acylation of oligosaccharides catalyzed by the metalloprotease thermolysin". Tetrahedron *62*(10): 2361–2369.

Pérez-Victoria, I., & Morales, J. C. (2007). "One- and two-step enzymatic synthesis of polymerizable vinyladipoyl mono- and diesters of non-reducing trisaccharides". Journal of Molecular Catalysis B: Enzymatic *48*(1–2): 8–15.

Pietinen, P., Rimm, E. B., Korhonen, P., Hartman, A. M., Willett, W. C., Albanes, D., & Virtamo, J. (1996). "Intake of Dietary Fiber and Risk of Coronary Heart Disease in a Cohort of Finnish Men". Circulation *94*(11): 2720–2727.

Plou, F. J., Cruces, M. A., Ferrer, M., Fuentes, G., Pastor, E., Bernabé, M., … Ballesteros, A. (2002). "Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent". Journal of Biotechnology *96*(1): 55–66.

Popper, Z. A., & Fry, S. C. (2008). "Xyloglucan-pectin linkages are formed intra-protoplasmically, contribute to wall-assembly, and remain stable in the cell wall". Planta *227*(4): 781–794.

Prabasari, I., Pettolino, F., Liao, M.-L., & Bacic, A. (2011). "Pectic polysaccharides from mature orange (Citrus sinensis) fruit albedo cell walls: Sequential extraction and chemical characterization". Carbohydrate Polymers *84*(1): 484–494.

Prade, R. A., Zhan, D., Ayoubi, P., & Mort, A. J. (1999). "Pectins, Pectinases and Plant-Microbe Interactions". Biotechnology and Genetic Engineering Reviews *16*(1): 361–392.

Rabelo, M. C., Honorato, T. L., Gonçalves, L. R. B., Pinto, G. A. S., & Rodrigues, S. (2006). "Enzymatic Synthesis of Prebiotic Oligosaccharides". Applied Biochemistry and Biotechnology *133*(1): 31–40.

Rabelo, M. C., Honorato, T. L., Gonçalves, L. R. B., Pinto, G. A. S., & Rodrigues, S. (2009). "Optimization of enzymatic synthesis of isomalto-oligosaccharides production". Journal of Food Biochemistry *33*(3): 342–354.

Ralet, M.-C., Thibault, J.-F., Faulds, C. B., & Williamson, G. (1994). "Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp". Carbohydrate Research *263*(2): 227–241.

Rana, V., Kumar, V., & Soni, P. L. (2009). "Structure of the oligosaccharides isolated from Dalbergia sissoo Roxb. leaf polysaccharide". Carbohydrate Polymers *78*(3): 520–525.

Rapaille, A., Gonze, M., & Van der Schueren, F. (1995). "Formulating Sugar-Free Chocolate Products with Maltitol". Food Technology *49*(7): 51–54.

Rehman, H., Hellweg, P., Taras, D., & Zentek, J. (2008). "Effects of Dietary Inulin on the Intestinal Short Chain Fatty Acids and Microbial Ecology in Broiler Chickens as Revealed by Denaturing Gradient Gel Electrophoresis". Poultry Science *87*(4): 783–789.

Reis, A., Domingues, M. R. M., Ferrer-Correia, A. J., & Coimbra, M. A. (2003). "Structural characterisation by MALDI-MS of olive xylo-oligosaccharides obtained by partial acid hydrolysis". Carbohydrate Polymers *53*(1): 101–107.

Reitsma, J. C. E., Thibault, J. F., & Pilnik, W. (1986). "Properties of amidated pectins. I. Preparation and characterization of amidated pectins and amidated pectic acids". Food Hydrocolloids *1*(2): 121–127.

Renard, C. M. G. C., & Thibault, J.-F. (1993). "Structure and properties of apple and sugar-beet pectins extracted by chelating agents". Carbohydrate Research *244*(1): 99–114.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). "Pectins: structure, biosynthesis, and oligogalacturonide-related signaling". Phytochemistry *57*(6): 929–967.

Rose, J. (2003). *The plant cell wall*. Blackwell (Oxford).

Rosell, K.-G., & Svensson, S. (1975). "Studies of the distribution of the 4-O-methyl-d-glucuronic acid residues in birch xylan". Carbohydrate Research *42*(2): 297–304.

Rosenquist, J., & Morrison, J. (1988). "The development of the cuticle and epicuticular wax of the grape berry". Vitis *27*: 63–70.

Rozi, P., Abuduwaili, A., Mutailifu, P., Gao, Y., Rakhmanberdieva, R., Aisa, H. A., & Yili, A. (2019). "Sequential extraction, characterization and antioxidant activity of polysaccharides from Fritillaria pallidiflora Schrenk". International Journal of Biological Macromolecules *131*: 97–106.

Rudjito, R. C., Ruthes, A. C., Jiménez-Quero, A., & Vilaplana, F. (2019). "Feruloylated Arabinoxylans from Wheat Bran: Optimization of Extraction Process and Validation at Pilot Scale". ACS Sustainable Chemistry & Engineering *7*(15): 13167–13177.

Saehu, S., Srisimarat, W., Prousoontorn, M. H., & Pongsawasdi, P. (2013). "Transglucosylation reaction of amylomaltase for the synthesis of anticariogenic oligosaccharides". Journal of Molecular Catalysis B: Enzymatic *88*: 77–83.

Sajilata, M. G., Singhal, R. S., & Kulkarni, P. R. (2006). "Resistant Starch - A Review". Comprehensive Reviews in Food Science and Food Safety *5*: 1–17.

Samanta, A. K., Senani, S., Kolte, A. P., Sridhar, M., Sampath, K. T., Jayapal, N., & Devi, A. (2012). "Production and in vitro evaluation of xylooligosaccharides generated from corn cobs". Food and Bioproducts Processing *90*(3): 466–474.

Sato, N., Takano, Y., Mizuno, M., Nozaki, K., Umemura, S., Matsuzawa, T., … Makishima, S. (2013). "Production of feruloylated arabino-oligosaccharides (FA-AOs) from beet fiber by hydrothermal treatment". The Journal of Supercritical Fluids *79*: 84–91.

Schindler, T. M. (1998). "The new view of the primary cell wall". Zeitschrift Für Pflanzenernährung Und Bodenkunde *161*(5): 499–508.

Schols, H. A., Bakx, E. J., Schipper, D., & Voragen, A. G. J. (1995). "A xylogalacturonan subunit present in the modified hairy regions of apple pectin". Carbohydrate Research *279*: 265–279.

Schröder, R., Nicolas, P., Vincent, S. J. ., Fischer, M., Reymond, S., & Redgwell, R. J. (2001). "Purification and characterisation of a galactoglucomannan from kiwifruit (Actinidia deliciosa)". Carbohydrate Research *331*(3): 291–306.

Sengkhamparn, N., Verhoef, R., Schols, H. A., Sajjaanantakul, T., & Voragen, A. G. J. (2009). "Characterisation of cell wall polysaccharides from okra (Abelmoschus esculentus (L.) Moench)". Carbohydrate Research *344*(14): 1824–1832.

Silva, J. L., Marroquin, E., Matta, F. B., Garner, J. O., & Stojanovic, J. (2005). "Physicochemical, carbohydrate and sensory characteristics of highbush and rabbiteye blueberry cultivars". Journal of the Science of Food and Agriculture *85*(11): 1815–1821.

Sims, I. M., Craik, D. J., & Bacic, A. (1997). "Structural characterisation of galactoglucomannan secreted by suspension-cultured cells of Nicotiana plumbaginifolia". Carbohydrate Research *303*(1): 79–92.

Smiricky-Tjardes, M. R., Grieshop, C. M., Flickinger, E. A., Bauer, L. L., & Fahey, G. C. J. (2003). "Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations , and ileal fermentative characteristics of growing pigs". Journal of Animal Science *81*(10): 2535–2545.

Smith, A., Nobmann, P., Henehan, G., Bourke, P., & Dunne, J. (2008). "Synthesis and antimicrobial evaluation of carbohydrate and polyhydroxylated non-carbohydrate fatty acid ester and ether derivatives.". Carbohydrate Research *343*(15): 2557–66.

Stückrath, R., Pedrero, A., Tarabla, P., & Trujillo, L. (1998). "Cuantificación y caracterización de las pectinas de cranberry (Vaccinium macrocarpon)". Alimentaria (293): 23–26.

Sucharipa, R. (1924). "PROTOPECTIN AND SOME OTHER CONSTITUENTS OF LEMON PEEL". Journal of the American Chemical Society *46*(1): 145–156.

Sun, J., Marais, J. P. J., Khoo, C., LaPlante, K., Vejborg, R. M., Givskov, M., … Rowley, D. C. (2015). "Cranberry (Vaccinium macrocarpon) oligosaccharides decrease biofilm formation by uropathogenic Escherichia coli". Journal of Functional Foods *17*: 235–242.

Taggart, P. (2004). *Starch in Food*. *Starch in Food*. Elsevier.

Tamayo-Cabezas, J., & Karboune, S. (2019). "Immobilized feruloyl esterase from Humicola insolens catalyzes the synthesis of feruloylated oligosaccharides". Process Biochemistry *79*: 81–90.

Tan, H., Chen, W., Liu, Q., Yang, G., & Li, K. (2018). "Pectin Oligosaccharides Ameliorate Colon Cancer by Regulating Oxidative Stress- and Inflammation-Activated Signaling Pathways". Frontiers in Immunology *9*: 1504.

Tan, L., Eberhard, S., Pattathil, S., Warder, C., Glushka, J., Yuan, C., … Mohnen, D. (2013). "An Arabidopsis Cell Wall Proteoglycan Consists of Pectin and Arabinoxylan Covalently Linked to an Arabinogalactan Protein". The Plant Cell *25*(1): 270–287.

Tanaka, M., Nakajima, Y., Nishio, K., & Hashimoto, H. (1993). "Structure of Oligosaccharides Prepared by Acidic Condensation of Palatinose". Journal of Carbohydrate Chemistry *12*(1): 49–61.

Tester, R. F., & Al-Ghazzewi, F. H. (2013). "Mannans and health, with a special focus on glucomannans". Food Research International *50*(1): 384–391.

Tingirikari, J. M. R. (2019). "In-Vitro Prebiotic Analysis of Microbiota Accessible Pectic Polysaccharides". Current Microbiology *76*(12): 1452–1460.

Topakas, E., Vafiadi, C., Stamatis, H., & Christakopoulos, P. (2005). "Sporotrichum thermophile type C feruloyl esterase (StFaeC): purification, characterization, and its use for phenolic acid (sugar) ester synthesis". Enzyme and Microbial Technology *36*(5–6): 729–736.

Tufenkji, N., Rifai, O. J., Harmidy, K., & Eydelnant, I. A. (2010). "Cranberry derived proanthocyanidins can prevent pathogen invasion of kidney epithelial cells". Food Research International *43*(3): 922–924.

Vafiadi, C., Topakas, E., Bakx, E. J., Schols, H. A., & Christakopoulos, P. (2007). "Structural Characterisation by ESI-MS of Feruloylated Arabino-oligosaccharides Synthesised by Chemoenzymatic Esterification". Molecules *12*(7): 1367–1375.

Van den Ende, W., De Roover, J., & Van Laere, A. (1996). "In vitro syethesis of fructofuranosyl-only oligosaccliarides from inulin and fructose by purified chicory root fructan:fructan fructosyl transferase". Physiologia Plantarum *97*(2): 346–352.

Van Laere, K. M. J., Hartemink, R., Bosveld, M., Schols, H. A., & Voragen, A. G. J. (2000). "Fermentation of Plant Cell Wall Derived Polysaccharides and Their Corresponding Oligosaccharides by Intestinal Bacteria". Journal of Agricultural and Food Chemistry *48*(5): 1644–1652.

Vandevelde, M.-C., & Fenyo, J.-C. (1985). "Macromolecular distribution of Acacia senegal gum (gum arabic) by size-exclusion chromatography". Carbohydrate Polymers *5*(4): 251–273.
Vattem, D. A., & Shetty, K. (2002). "SOLID-STATE PRODUCTION OF PHENOLIC ANTIOXIDANTS FROM CRANBERRY POMACE BY RHIZOPUS OLIGOSPORUS". Food Biotechnology *16*(3): 189–210.
Vázquez-Rodríguez, B., Gutiérrez-Uribe, J. A., Antunes-Ricardo, M., Santos-Zea, L., & Cruz-Suárez, L. E. (2020). "Ultrasound-assisted extraction of phlorotannins and polysaccharides from Silvetia compressa (Phaeophyceae)". Journal of Applied Phycology *32*(2): 1441–1453.
Vicente, A. R., Ortugno, C., Rosli, H., Powell, A. L. T., Greve, L. C., & Labavitch, J. M. (2007). "Temporal Sequence of Cell Wall Disassembly Events in Developing Fruits. 2 . Analysis of Blueberry (Vaccinium Species)". Journal of Agricultural and Food Chemistry *55*(10): 4125–4130.
Vierhuis, E., Schols, H. A., Beldman, G., & Voragen, A. G. J. (2000). "Isolation and characterisation of cell wall material from olive fruit (Olea europaea cv koroneiki) at different ripening stages". Carbohydrate Polymers *43*(1): 11–21.
Vierhuis, E., York, W. S., Kolli, V. S. K., Vincken, J.-P., Schols, H. A., Van Alebeek, G.-J. W. ., & Voragen, A. G. J. (2001). "Structural analyses of two arabinose containing oligosaccharides derived from olive fruit xyloglucan: XXSG and XLSG". Carbohydrate Research *332*(3): 285–297.
Vigsnæs, L. K., Holck, J., Meyer, A. S., & Licht, T. R. (2011). "In Vitro Fermentation of Sugar Beet Arabino-Oligosaccharides by Fecal Microbiota Obtained from Patients with Ulcerative Colitis To Selectively Stimulate the Growth of Bifidobacterium spp. and Lactobacillus spp.". Applied and Environmental Microbiology *77*(23): 8336–8344.
Vithanage, C. R., Grimson, M. J., Wills, P. R., Harrison, P., & Smith, B. G. (2010). "Rheological and Structural Properties of High-Methoxyl Esterified, Low-Methoxyl Esterified and Low-Methoxyl Amidated Pectin Gels". Journal of Texture Studies *41*(6): 899–927.
Vodenicarova, M., Drimalova, G., Hromadkova, Z., Malovikova, A., & Ebringerova, A. (2006). "Xyloglucan degradation using different radiation sources: A comparative study". Ultrasonics Sonochemistry *13*(2): 157–164.
Voicu, S. I., Condruz, R. M., Mitran, V., Cimpean, A., Miculescu, F., Andronescu, C., … Thakur, V. K. (2016). "Sericin Covalent Immobilization onto Cellulose Acetate Membrane for Biomedical Applications". ACS Sustainable Chemistry & Engineering *4*(3): 1765–1774.
Wang, Q., Ying, T., Jahangir, M. M., & Jiang, T. (2012). "Study on removal of coloured impurity in soybean oligosaccharides extracted from sweet slurry by adsorption resins". Journal of Food Engineering *111*(2): 386–393.
Wang, X., & Lü, X. (2014). "Characterization of pectic polysaccharides extracted from apple pomace by hot-compressed water.". Carbohydrate Polymers *102*: 174–84.
White, B. L., Howard, L. R., & Prior, R. L. (2010a). "Polyphenolic Composition and Antioxidant Capacity of Extruded Cranberry Pomace". Journal of Agricultural and Food Chemistry *58*(7): 4037–4042.
White, B. L., Howard, L. R., & Prior, R. L. (2010b). "Proximate and Polyphenolic Characterization of Cranberry Pomace". Journal of Agricultural and Food Chemistry *58*(7): 4030–4036.
Woo, A. H., Elbe, J. H., & Amundson, C. H. (1980). "Anthocyanin Recovery from Cranberry Pulp Wastes by Membrane Technology". Journal of Food Science *45*(4): 875–879.
Wu, J., Wu, Y., Yang, C., & Wang, Z. (2012). "Enzymatic Preparation and Characterization of Soybean Oligosaccharides from Okara". Procedia Engineering *37*: 186–191.
Xu, G., Amicucci, M. J., Cheng, Z., Galermo, A. G., & Lebrilla, C. B. (2018). "Revisiting monosaccharide analysis – quantitation of a comprehensive set of monosaccharides using dynamic

multiple reaction monitoring". The Analyst *143*(1): 200–207.
Yang, B., Prasad, K. N., Xie, H., Lin, S., & Jiang, Y. (2011). "Structural characteristics of oligosaccharides from soy sauce lees and their potential prebiotic effect on lactic acid bacteria". Food Chemistry *126*(2): 590–594.
Yapo, B. M. (2011). "Rhamnogalacturonan-I: A Structurally Puzzling and Functionally Versatile Polysaccharide from Plant Cell Walls and Mucilages". Polymer Reviews *51*(4): 391–413.
Yen, G.-C., & Lin, H.-T. (1998). "High pressure and heat treatments effects on pectic substances in guava juice". In F. Shahidi, C.-T. Ho, & N. van Chuyen (Eds.), *Process-Induced Chemical Changes in Food* (pp. 81–90). Plenum Press (New York).
Yin, B., Deng, W., Xu, K., Huang, L., & Yao, P. (2012). "Stable nano-sized emulsions produced from soy protein and soy polysaccharide complexes". Journal of Colloid and Interface Science *380*(1): 51–59.
Ying, Z., Han, X., & Li, J. (2011). "Ultrasound-assisted extraction of polysaccharides from mulberry leaves.". Food Chemistry *127*(3): 1273–9.
Yokoyama, W. H., Hudson, C. A., Knuckles, B. E., Chiu, M.-C. M., Sayre, R. N., Turnlund, J. R., & Schneeman, B. O. (1997). "Effect of Barley β-Glucan in Durum Wheat Pasta on Human Glycemic Response". Cereal Chemistry *74*: 293–296.
Yoo, S.-H., Lee, B.-H., Lee, H., Lee, S., Bae, I. Y., Lee, H. G., … Hotchkiss Jr., A. T. (2012). "Structural Characteristics of Pumpkin Pectin Extracted by Microwave Heating". Journal of Food Science *77*(11): C1169–C1173.
Yoshida-Shimokawa, T., Yoshida, S., Kakegawa, K., & Ishii, T. (2001). "Enzymic feruloylation of arabinoxylan-trisaccharide by feruloyl-CoA:arabinoxylan-trisaccharide O -hydroxycinnamoyl transferase from Oryza sativa". Planta *212*(3): 470–474.
Yu, L., Yu, C., Zhu, M., Cao, Y., Yang, H., Zhang, X., … Zhou, G. (2015). "Structural analysis of galactoarabinan from duckweed". Carbohydrate Polymers *117*: 807–812.
Zamil, M. S., & Geitmann, A. (2017). "The middle lamella—more than a glue". Physical Biology *14*(1): 015004.
Zandleven, J., Beldman, G., Bosveld, M., Schols, H. A., & Voragen, A. G. J. (2006). "Enzymatic degradation studies of xylogalacturonans from apple and potato, using xylogalacturonan hydrolase". Carbohydrate Polymers *65*(4): 495–503.
Zandleven, Joris, Sørensen, S. O., Harholt, J., Beldman, G., Schols, H. A., Scheller, H. V., & Voragen, A. G. J. (2007). "Xylogalacturonan exists in cell walls from various tissues of Arabidopsis thaliana". Phytochemistry *68*(8): 1219–1226.
Zhang, Y.-H. P., & Lynd, L. R. (2004). "Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems.". Biotechnology and Bioengineering *88*(7): 797–824.
Zhu, R., Hong, M., Zhuang, C., Zhang, L., Wang, C., Liu, J., … Chen, G. (2019). "Pectin oligosaccharides from hawthorn (Crataegus pinnatifida Bunge. Var. major) inhibit the formation of advanced glycation end products in infant formula milk powder". Food & Function *10*(12): 8081–8093.
Zilliox, C., & Debeire, P. (1998). "Hydrolysis of wheat straw by a thermostable endoxylanase: Adsorption and kinetic studies". Enzyme and Microbial Technology *22*(1): 58–63.
Zykwinska, A. W., Ralet, M.-C. J., Garnier, C. D., & Thibault, J.-F. J. (2005). "Evidence for In Vitro Binding of Pectin Side Chains to Cellulose". Plant Physiology *139*(1): 397–407.
Zykwinska, A. W., Rondeau-Mouro, C., Garnier, C., Thibault, J.-F., & Ralet, M.-C. (2006). "Alkaline extractability of pectic arabinan and galactan and their mobility in sugar beet and potato cell walls". Carbohydrate Polymers *65*(4): 510–520.