Characterization of sugar beet pulp derived oligosaccharides

Martijn Leijdekkers

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Thesis

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Voor Floortje, Suze en Mats

Abstract

This thesis aimed at characterizing complex mixtures of sugar beet pulp derived oligosaccharides, in order to be able to monitor and optimize the enzymatic saccharification of sugar beet pulp.

Hydrophilic interaction chromatography with on-line evaporative light scattering detection and multidimensional mass spectrometry (HILIC-ELSD-MS^n) was developed as a versatile technique for the characterization of a wide range of neutral and acidic plant cell wall derived oligosaccharides. It was shown that the separation capacity of HILIC for acidic oligosaccharides outperforms other techniques. HILIC-MS^n enabled efficient sequence elucidation of oligosaccharides in complex mixtures.

The enzymatic saccharification of sugar beet pulp was optimized to release the maximum amounts of monomeric galacturonic acid and arabinose with limited concomitant degradation of cellulose, using conditions that are feasible for industrial upscaling. The oligosaccharides that were obtained after hydrolysis were characterized, thereby enabling recognition of enzyme activities additionally needed for the full degradation of recalcitrant oligosaccharides.

The *in vitro* fermentation characteristics of sugar beet pectic oligosaccharides (SBPOS) were studied using human and pig fecal inocula. The fate of the different classes of SBPOS, the production of short-chain fatty acids and the changes in human fecal bacterial populations during *in vitro* fermentation were described. Several modifications in the microbiota composition that are potentially beneficial to host health were observed.

HILIC was also coupled to traveling-wave ion mobility mass spectrometry to enable the simultaneous separation and characterization of complex mixtures of various isomeric pectic oligosaccharides. The developed method was used to characterize isomeric sugar beet rhamnogalacturonan I derived oligosaccharides carrying a glucuronic acid substituent, thereby identifying novel structural features of sugar beet pectin.

List of abbreviations

3-AQ	3-aminoquinoline
Ac	Acetyl
ACN	Acetonitrile
Ara	Arabinose
CCS	Collision cross section
CE	Capillary electrophoresis
DA	Degree of acetylation
DHB	2,5-dihydroxybenzoic acid
DM	Degree of methyl esterification
DP	Degree of polymerization
ELSD	Evaporative light scattering detection
ESI	Electrospray ionization
FLR	Fluorescence
FOS	Fructo-oligosaccharides
Gal	Galactose
GalA	Galacturonic acid
GlcA	Glucuronic acid
HG	Homogalacturonan
HILIC	Hydrophilic interaction chromatography
HPAEC	High-performance anion exchange chromatography
HPLC	High-performance liquid chromatography
HPSEC	High-performance size exclusion chromatography
LIF	Laser-induced fluorescence
MALDI–TOF	Matrix-assisted laser desorption/ionization–time-of-flight
MALLS	Multi-angle laser light scattering
Me	Methyl
MES	2-(*N*-morpholino)ethanesulfonic acid
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PAD	Pulsed amperometric detection
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
Rha	Rhamnose
RI	Refractive index
RP	Reversed phase
SBPOS	Sugar beet pectic oligosaccharides
SCFA	Short-chain fatty acids
SIEM	Standard ileal efflux medium
TFA	Trifluoroacetic acid
TWIMS	Traveling-wave ion mobility spectrometry
Xyl	Xylose

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Chapter 1

General introduction

1.1 Project outline

Sugar beet pulp is the cell debris that is obtained after sugar extraction from sliced sugar beet (*Beta vulgaris* L.) roots. It is a large volume by-product of the beet sugar industry. In Europe, annually 5 to 6 million tons of sugar beet pulp remain from sugar production on dry matter basis *[1]*. Currently, its main use is animal feed *[2]*. Because of this, its economic value is relatively low. Therefore, it is worthwhile to search for opportunities towards increasing the economic value of sugar beet pulp.
This PhD-thesis is part of an industrial project that aims to develop a biorefinery process for the conversion of sugar beet pulp into multiple value-added 'green' products. Hereto, an industrial process is under development that enables the release and subsequent purification of different value-added components from sugar beet pulp. The process comprises the controlled enzymatic degradation of the cell wall polysaccharides present in sugar beet pulp to release different mono- and oligosaccharides. Within this industrial project, this research focuses on characterizing the complex mixtures of oligosaccharides that are obtained after enzymatic saccharification, thereby enabling identification of lacking enzyme activities for further release of valuable monosaccharides from sugar beet pulp. In addition, the potential of the recalcitrant sugar beet pulp derived oligosaccharides to be utilized as functional food or feed ingredients is explored by studying their *in vitro* fermentability.

1.2 Sugar beet pulp composition

1.2.1 Average chemical composition

Sugar beet pulp is mainly composed of the water-insoluble plant cell wall material that remains after aqueous extraction of sucrose from sugar beet roots in the factory. Its precise composition is determined by agronomic factors, such as sugar beet variety, origin, developmental stage of the beet tissue and factory processing conditions. It is mainly available as pressed pulp after sucrose extraction with a relatively low dry matter content of 20-25% (w/w) *[1]*.
The average chemical composition of sugar beet pulp is shown in Table 1.1. On dry matter basis, sugar beet pulp consists up to 80% of carbohydrates. The main constituent monosaccharides present in sugar beet pulp are glucose (from cellulose), arabinose and uronic acid (both from pectin). They already constitute 60-70% of the dry matter of sugar beet pulp. About 2-3% residual sucrose is also present. Besides carbohydrates, sugar beet pulp contains 7.0-13.2% of protein and low levels of minerals, lignin and lipids. The lignin content of sugar beet pulp is low,

1.8-3.4%, compared to other agricultural by-products, making it an interesting feedstock for biorefinery *[3]*.

Table 1.1. Average composition of sugar beet pulp.

Fraction	% (w/w)†	References
Glucose‡	21.1-24.5	*[4-7]*
Arabinose‡	17.3-23.5	*[4-7]*
Galactose‡	4.3-5.8	*[4-7]*
Rhamnose‡	1.1-2.4	*[4-7]*
Uronic acid‡	18.0-22.0	*[4-8]*
Ferulic acid‡	0.5-0.9	*[4-7]*
Xylose‡	1.1-1.7	*[4-7]*
Mannose‡	1.0-1.5	*[4-7]*
Fucose‡	0.1-0.2	*[4, 7]*
Methanol‡	0.4-2.3	*[4-7]*
Acetic acid ‡	1.6-3.9	*[4-7]*
Sucrose	2.1-3.0	*[6, 9]*
Protein (N×6.25)	7.0-13.2	*[4, 5, 7-11]*
Lignin	1.8-3.4	*[5, 8, 10]*
Fat	1.0-2.0	*[8, 9]*
Ash	3.6-8.4	*[4, 5, 9-11]*

† dry matter basis
‡ sugar beet cell wall polysaccharide constituents

1.2.2 Sugar beet cell wall

1.2.2.1 Plant cell wall architecture

The plant cell wall is a complex polysaccharide network that surrounds the plant cell. The cell wall not only strengthens the plant body, but also has key roles in plant growth, cell differentiation, intercellular communication, water transport and defence *[12]*. The composition of the cell wall changes during different developmental stages of a plant and varies for different parts of the plant *[13]*. Figure 1.1 shows a simplified model of plant cells and the plant cell wall architecture *[14]*.

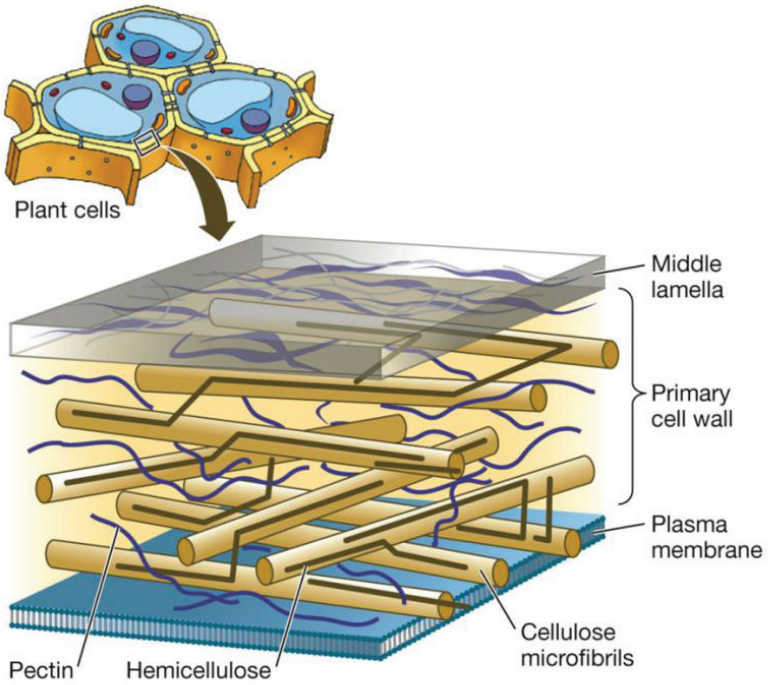

Figure 1.1. Schematic representation of plant cells and the plant cell wall architecture. Adapted from *[14]*.

The primary cell wall predominantly consists of cellulose, which is embedded in a matrix of pectin and hemicellulose. Besides this network of polysaccharides, proteins and lignin are also part of the cell wall *[15, 16]*. In general, there are two types of primary cell walls. Type I primary cell walls are found in dicotyledons (e.g. sugar beet), non-graminaceous monocotyledons and gymnosperms and typically contain pectin and xyloglucan *[17]*. Type II primary cell walls are found in the Poaceae or Gramineae (grains) and are rich in hemicelluloses, mainly arabinoxylan. In contrast to type I, type II primary cell walls only contain small amounts (<10%) of pectin *[18]*. Pectins can be covalently linked to each other and covalent crosslinks and/or hydrogen bonds between pectin and hemicellulose and between pectin and cellulose have been described *[19, 20]*.

1.2.2.2 Sugar beet cell wall polysaccharides

The cell wall of sugar beet roots differs from normal type I primary cell walls as it is almost devoid of xyloglucan and mostly consists of cellulose and pectin *[21]*.

Cellulose

Cellulose is a linear homopolymer composed of β-(1,4)-linked D-glucose units (Figure 1.2). These polymers aggregate and form microfibrils via hydrogen bonds *[22]*. The microfibrils form a highly ordered crystalline arrangement or a less ordered amorphous region *[23]*. Depending on the isolation method, molecular masses of sugar beet cellulose range from 300 to 900 kDa *[24]*.

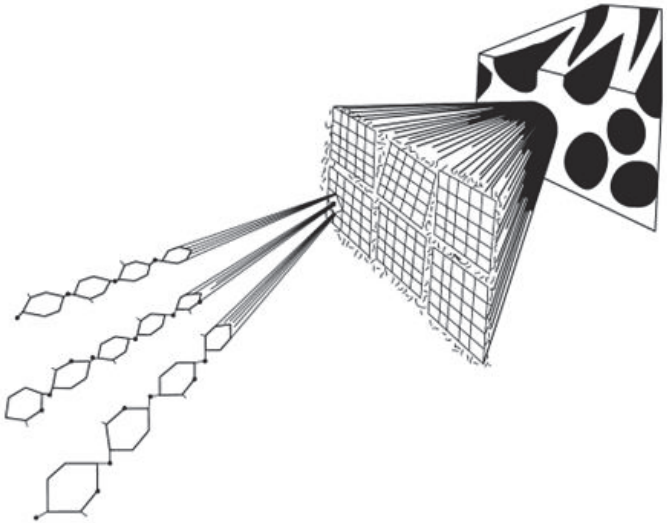

Figure 1.2. Schematic representation of cellulose microfibrils and molecular structure of cellulose. Adapted from *[25]*.

Pectin

The molecular structure of sugar beet pectin has been described extensively *[e.g., 26, 27]*. It is a complex polysaccharide that consists of several structural elements: Homogalacturonan (HG), rhamnogalacturonan I (RG I) and, to a much lesser extent, rhamnogalacturonan II (RG II) regions. It is still a matter of debate how these structural elements are connected to each other to form the macromolecular structure. Three pectin structural models are considered nowadays, as illustrated in Figure 1.3: The *"smooth and hairy regions"* model *[28]*, the *"side chain model" [29]* and the *"combined side chain-hairy regions model" [30, 31]*. The "smooth and hairy regions model" suggests a backbone structure of "smooth" regions of HG, alternating with "hairy" regions of RG I that contain neutral sugars side chains. In the "side chain model", HG is present as side chains of a backbone of RG I. In the

"combined side chain-hairy regions" model, HG is both present in the pectin backbone, alternating with RG I, and as side chains of RG I.

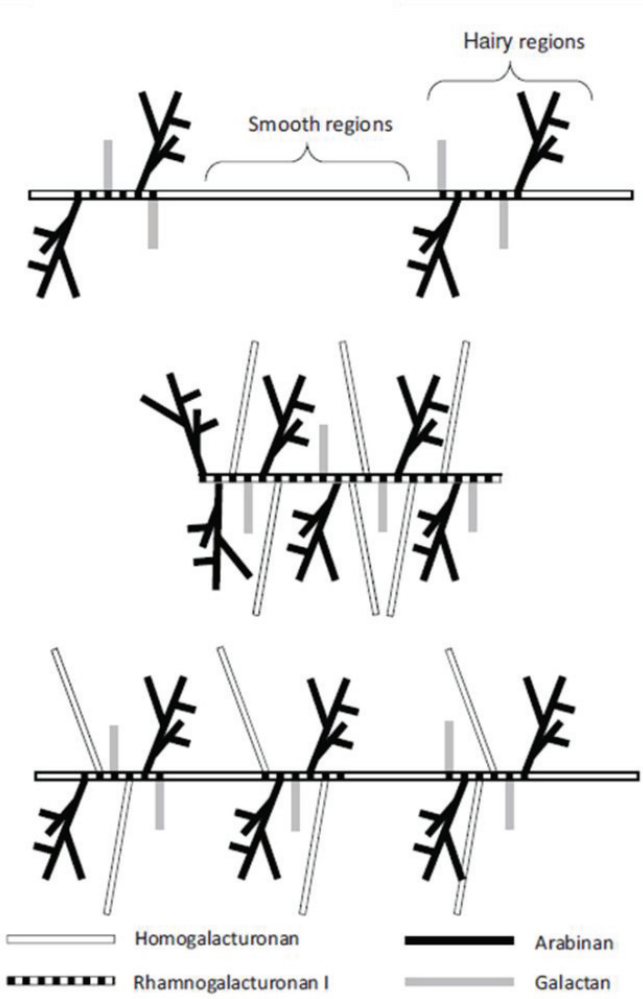

Figure 1.3. Schematic representation of the three different pectin models. Upper: smooth and hairy regions model, middle: side chain model, lower: combined side chain-hairy regions model. Adapted from *[6]*.

Figure 1.4 shows a schematic representation of the different sugar beet pectin structural elements. HG consists of α-(1,4)-linked D-galacturonic acid residues. The galacturonic acid residues can be methyl esterified at the C6 position and acetylation can occur at *O*-2 and/or *O*-3. The number of methyl esters and acetyl groups per 100 galacturonic acid residues determines the degree of methyl esterification (DM) and the degree of acetylation (DA), respectively. It was reported that acid extracted sugar beet pectin can have a DM of 62 and a DA of 30 *[32]*. Stretches of HG with galacturonic acid residues that are not methyl esterified can associate via calcium crosslinking *[33]*. The distribution of acetyl groups in HG of sugar beet pectin has been proposed to be blockwise *[34]*. It has been estimated that about 75% of all acetyl groups are located in HG and 25% in RG I of sugar beet pectin *[35]*.

RG I is composed of a backbone of alternating galacturonic acid and rhamnose residues. Linear β-(1,4)-linked galactan and highly branched arabinan, composed of α-(1,5)-linked backbones with α-(1,2)- and/or α-(1,3)-arabinofuranosyl substitutions, are side chains of RG I. The galactan and arabinan side chains of sugar beet pectin RG I can be feruloylated. Most of the ferulic acid residues are present as monomers, but various types of dimers may be present, thereby forming crosslinks between arabinan and galactan side chains *[36, 37]*. The galacturonic acid residues in the RG I backbone can be acetylated at *O*-2 and/or *O*-3 *[38]*. Presence of galacturonic acid residues in RG I, which are substituted at *O*-3 with glucuronic acid have also been reported for sugar beet pectin. It was estimated that less than one in fifty galacturonic acid residues in sugar beet RG I carries a glucuronic acid substituent, making this a rare structural feature *[39]*.

The highly complex RG II substructure, which is only present in minor amounts in sugar beet pectin, can be composed of at least 12 different monosaccharide residues, linked together by more than 20 different glycosidic linkages. RG II is able to form dimers via a borate diester, which results in a crosslink of two pectin molecules within the cell wall *[40]*.

Sugar beet pectin differs from pectin from other sources, such as citrus and apple: Its length of HG is shorter *[32]*, its amount of RG I is higher *[41]* and its DA, which is about 30%, is higher *[35]*. Acetyl groups are present in both HG and RG I. Sugar beet RG I is rich in arabinan side chains. The pectic arabinan content represents 20-25% of the sugar beet pulp dry matter *[36]*. The combination of these structural features contributes to the poor gelling properties of sugar beet pectin compared to those of pectin from citrus and apple *[32]*.

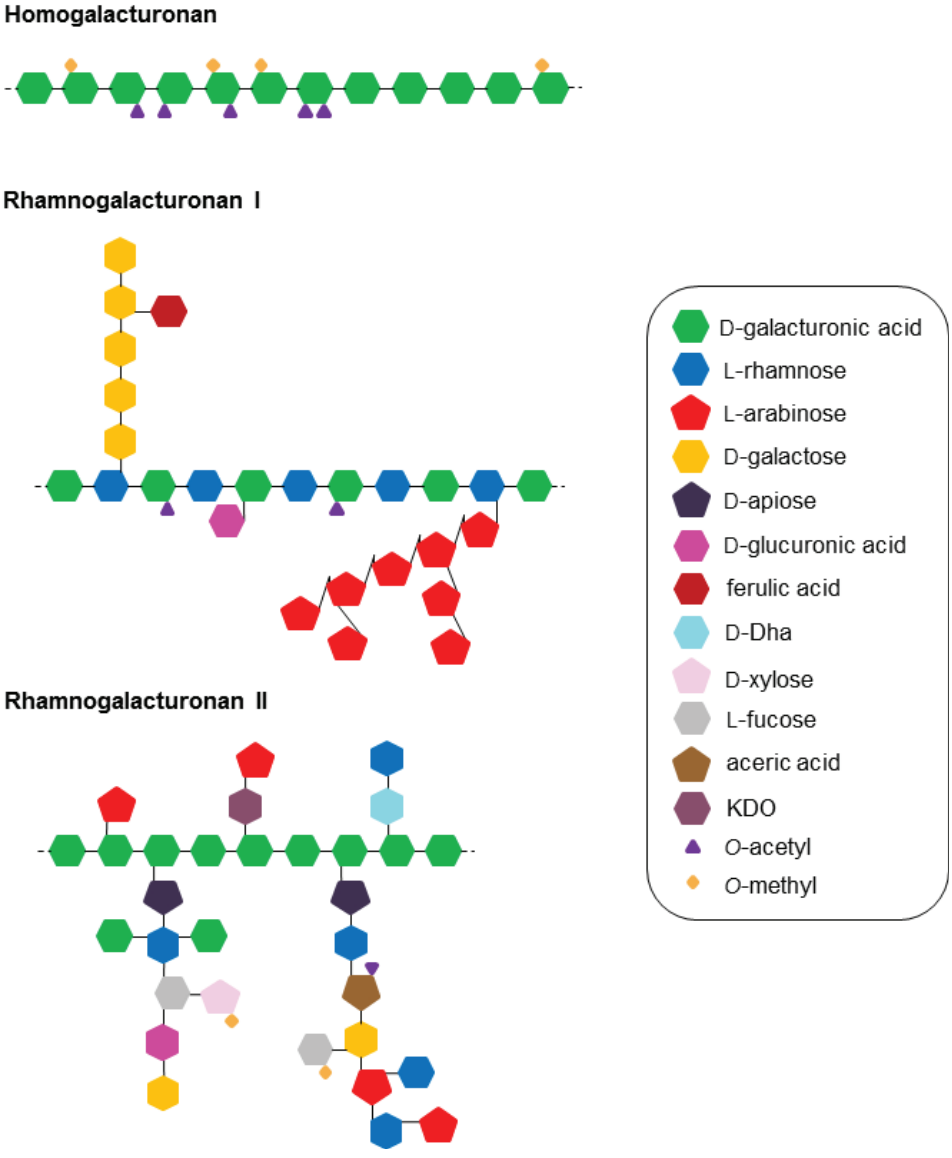

Figure 1.4. Schematic representation of the sugar beet pectin substructures homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II. Legend: D-Dha, 2-keto-3-deoxy-D-lyxo-heptulosaric acid; KDO, 2-keto-3-deoxy-D-manno-octulosonic acid. Adapted from *[42]*.

1.3 Sugar beet pulp valorization opportunities

As stated above, animal feed currently is the main use of sugar beet pulp. Nevertheless, the composition of sugar beet pulp suggests interesting alternatives. Several value-added products that can be produced from sugar beet pulp have been described and will be discussed in this section.

1.3.1 Application of sugar beet pulp as food ingredient

Sugar beet fiber is a product that is made from dried sugar beet pulp and is already commercially available *[43]*. It is sold as a functional dietary fiber and as an ingredient in baking, meat and other foods. To be used as a food ingredient, the production of sugar beet fiber has to meet the regulatory requirements for food production. The application of sugar beet fiber in a wide range of food products is limited because of its insolubility and high water binding capacity *[44]*.

1.3.2 Utilization of sugar beet pulp derived polysaccharides

Utilization of sugar beet pulp derived cellulose
The production of nano-scale cellulose fibers and their application in composite materials has gained increasing attention due to their high strength and stiffness combined with low weight, biodegradability and renewability *[45, 46]*. It has been demonstrated that cellulose nanofibers can be produced from de-pectinated sugar beet pulp using chemical treatments (alkali treatment and bleaching) and high-pressure homogenization *[47]*. Such cellulose nanofibers have shown excellent reinforcement properties in combination with a synthetic polymer matrix *[48]*.
Sugar beet derived cellulose may also be utilized for paper production as environmentally friendly additive for improvement of paper strength properties and as partial replacement of wood fibres, as has been described previously *[49]*. Another application of sugar beet pulp derived cellulose is its use as rheology modifier and thickener in liquid detergents and paints *[50]*.

Utilization of sugar beet pulp derived pectin
Pectin is commonly used as gelling and viscosifying agent in food products. The gelation mechanism of pectins is mainly governed by their degree of esterification. Thus far, no pectins have been extracted from sugar beet pulp with gelling and texturizing properties comparable to pectins extracted from citrus peels and apple pomace. The poor gelling ability was attributed to the higher DA, lower molecular weight and higher proportion of neutral side-chains in sugar beet pectin compared to pectin derived from citrus and apple. To improve its gelling properties, the

structure of sugar beet pectin has to be modified *[51]*. Enzymatic deacetylation could be a promising approach to achieve this *[52]*.
On the other hand, the high DA and the high content of neutral side-chains of commercial sugar beet pectin contributes to its emulsion functionality. It has been reported that sugar beet pectin is a much better citrus oil stabilizer in carbonated soft drinks compared to typical commercial citrus pectin *[53]*.

1.3.3 Utilization of sugar beet pulp derived oligosaccharides

Utilization as prebiotic ingredient
A prebiotic is defined as a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health *[54]*. The selective fermentation of a prebiotic by the anaerobic, colonic microbiota may lead to several beneficial effects. These effects include stimulation of the growth and/or activity of intestinal bacteria that are associated with host health (e.g., bifidobacteria and lactobacilli) and production of short chain fatty acids (SCFA) and lactate. These SCFA (mainly acetate, propionate and butyrate) and lactate lower the intracolonic pH, which inhibits the growth of pathogens (e.g., clostridia). The majority of SCFAs are absorbed from the gut and metabolized in the body, making a relatively small, but significant, contribution to the body's daily energy requirements *[55]*. Furthermore, SCFA have been shown to influence various processes in the body, including satiety, cholesterol synthesis and carcinogenesis *[56]*.
There is an increasing interest in the potential of generating prebiotic oligosaccharides from pectic materials, such as sugar beet pulp *[57, 58]*. Previous studies described tailored enzymatic production of specific classes of oligosaccharides from sugar beet pulp, e.g., arabino oligosaccharides *[59-61]* and oligosaccharides derived from HG and RG I, and demonstrated their prebiotic potential *[62]*. However, sufficient scientific evidence is required before a novel oligosaccharide can be accorded prebiotic status.
Ultimately, any claim to prebiotic status for a food or feed ingredient must be established by *in vivo* studies *[58]*. However, these human or animal studies are expensive and it may be questionable to feed human or animal subjects test ingredients whose effects during digestion are not yet fully understood. Therefore, it has become common practice to evaluate prebiotic candidates initially using *in vitro* fermentation studies *[58]*. *In vitro* methods have often been used to predict how certain dietary fibers may behave in the body and these methods have the advantage that individual dietary fibers can be tested in pure form without the interference of other compounds.

Previously, pure cultures of intestinal bacterial isolates were used for testing prebiotic candidates. However, this approach does not provide information on how a prebiotic candidate will behave in a mixed culture system, such as present in the colon. A more useful initial test for screening the prebiotic potential is the use of fecal inocula in batch cultures *[58]*. Testing in batch systems can be followed by evaluation in a more sophisticated dynamic *in vitro* model of the human gut, which simulates the average conditions in the lumen of the human proximal colon by controlling temperature, pH and peristaltic movements and removal of water and fermentation products by an automated dialysis system *[63]*.
Samples from *in vitro* fermentation studies are often analyzed for gases, short-chain fatty acids, substrate degradation and microbiota composition. Comparing these data with those obtained during *in vitro* fermentation of well-established prebiotics, such as fructo-oligosaccharides or galacto-oligosaccharides, enables to evaluate the prebiotic potential of novel dietary fibers *[58]*.

Utilization as plant protection products
Oligogalacturonic acids are produced *in planta* in response to the damage caused by invading pathogens, which trigger a variety of plant defence responses. There is evidence that oligogalacturonic acids are able to enhance non-host plant resistance against pathogens and it is thought that mimicking a pathogen attack with such non-specific elicitors could become an alternative strategy in crop plant protection *[64, 65]*. In addition, oligogalacturonic acids can also have growth-modulating properties *[66, 67]*. It has been described that the degree of methyl esterification as well as the degree of polymerization are determinant for the biological activity of oligogalacturonic acids *[68]*.
Currently, synthetic plant protection products provide the primary means for controlling plant diseases. Increasing public concern over the use of synthetic pesticides and proliferation of resistance in the pathogen populations require the development of alternative control strategies to reduce dependency on synthetic pesticides *[69]*. Therefore, it is useful to investigate the potential use of sugar beet pectin derived oligogalacturonic acids as natural plant protection products.

1.3.4 Utilization of sugar beet pulp derived monosaccharides

Sugar beet pulp is considered as a potential resource of carbohydrates for biorefinery, in particular biofuel production and the production of platform chemicals. Prior to such use, the carbohydrate polymers need to be hydrolyzed into their monosaccharide constituents *[6]*.

Conversion into bioethanol

Several studies have focused on the formation of monosaccharides from sugar beet pulp for their subsequent fermentation into ethanol *[3, 70-72]*. However, common yeasts can only ferment hexoses. Engineered yeast strains are needed for the fermentation of pentoses, such as arabinose or xylose *[73]*. Next to this, it has been described that galacturonic acid, constituting 18-22% of sugar beet pulp, strongly inhibits the fermentation of galactose by the yeast *Saccharomyces cerevisiae* (the main producer of bioethanol) and the fermentation of arabinose and xylose by an engineered pentose-fermenting *S. cerevisiae* strain *[74]*. Moreover, *S. cerevisiae* is unable to ferment galacturonic acid. Thus far, galacturonic acid fermentation has only been described in bacteria *[75]*. This makes the economics of ethanol production from sugar beet pulp challenging.

Conversion into biogas

It has been demonstrated that production of methane by anaerobic biodegradation of sugar beet pulp is feasible *[76, 77]*. In Europe, several anaerobic digesters are already in operation for production of biogas from sugar beet pulp *[1]*. However, because biogas is a low-grade product, the economic value of sugar beet pulp using this conversion process remains relatively low and still largely depends on green energy subsidies.

Conversion into platform chemicals / biobased building blocks

Instead of serving as carbon source for fermentation, the sugar beet pectin derived monosaccharides are interesting molecules for further conversion into building blocks, which can be subsequently transformed to different high-value biobased chemicals or materials. Figure 1.5 shows 12 top candidate molecules that have been previously identified. They can be potentially produced from biomass derived monosaccharides and act as chemical building blocks for the production of biobased materials *[78-80]*. Plant derived monosaccharides can be converted into these top chemical building block candidates by fermentation or (bio-)chemical catalysis *[80, 81]*. These building block molecules can be subsequently transformed into a wide range of high-value biobased materials, such as vinyl polymers, polyesters, polyamides or polyurethanes *[82]*. Currently, building blocks for condensation polymers, such as 2,5-furandicarboxylic acid (FDCA) attract considerable attention. This building block is increasingly promoted as a biobased alternative to terephtalic acid (TA) as recent reports have shown that FDCA-based polymers are in many aspects comparable or superior to their TA-analogues *[83, 84]*. It has been demonstrated recently that FDCA can be produced from aldaric acids which can be derived from pectin derived uronic acids *[83]*. Figure 1.6 shows a route from uronic acids to aldaric acids and subsequently derived products. This

provides an interesting opportunity for the valorization of sugar beet pulp derived galacturonic acid.

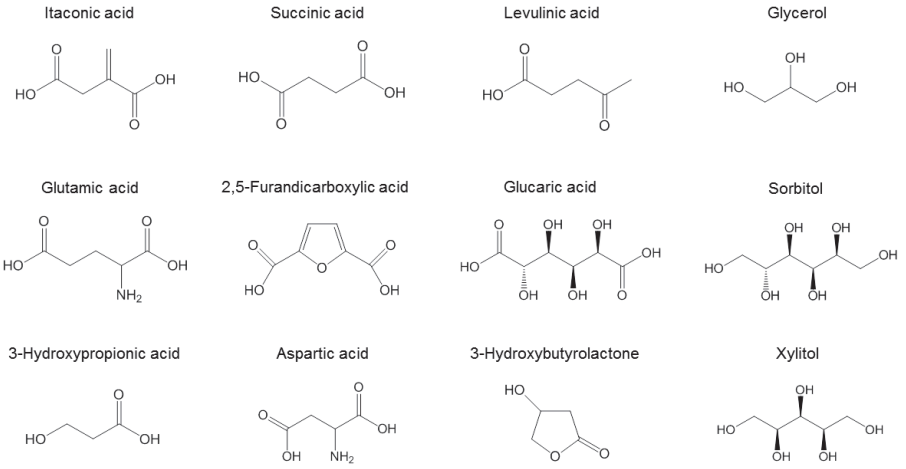

Figure 1.5. Top-12 molecules that could potentially act as building blocks for commodity chemicals and chemical intermediates *[80]*.

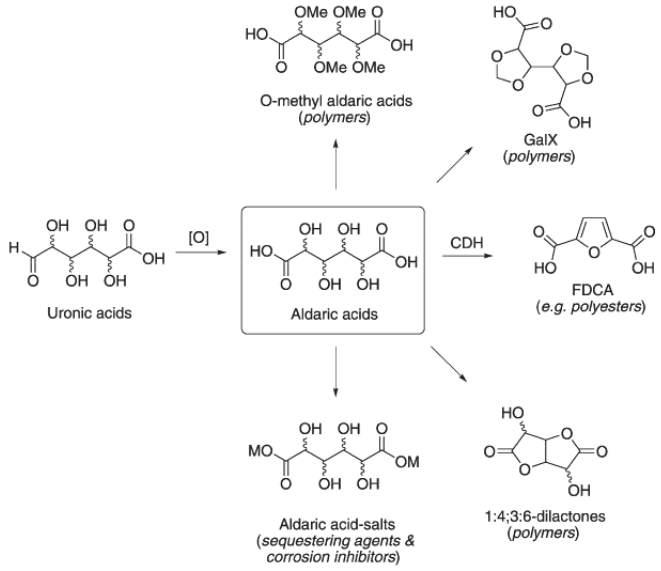

Figure 1.6. Route from uronic acids to aldaric acids, derived products and main applications. Legend: [O], oxidation; CDH, cyclodehydration *[83]*.

Utilization of arabinose as food ingredient
Instead of transformation of sugar beet pulp derived arabinose into platform chemicals, it may also be used as a food ingredient that is able to lower the glycemic index of a food product. It has been demonstrated that arabinose can act as a sucrase inhibitor during digestion in the small intestine, thereby lowering the blood glucose peak levels *[85, 86]*. Therefore, it might be interesting to investigate if arabinose can be used to develop food products with a low glycemic index or diabetic food formulations.

1.4 Saccharification of sugar beet pulp

To produce different value-added products from sugar beet pulp as outlined above, saccharification of the sugar beet polysaccharides is required. Saccharification is the degradation of polysaccharides into their constituent monosaccharides and can be performed either chemically, enzymatically or by a combination of both.

1.4.1 Chemical saccharification

Acid hydrolysis (using hydrochloric or sulfuric acid in concentrations up to 1.6 M) at temperatures of 80-100 °C has been described for the chemical saccharification of sugar beet pulp. Solubilization of sugar beet pulp up to 75% was achieved, but the polysaccharides were not completely degraded into monosaccharides *[87]*. In general, chemical saccharification results in high amounts of chemical waste (acid/salt), substantial energy consumption, unspecific degradation of polysaccharides and partial breakdown of the monosaccharides released *[87, 88]*. Therefore, chemical saccharification processes are usually undesired.

1.4.2 Enzymatic saccharification

Because of the drawbacks of chemical treatments, enzymatic saccharification is considered as a more favorable approach. Various enzymes are needed to fully degrade sugar beet cell wall polysaccharides. Enzymes involved in the degradation of carbohydrates are collectively designated as Carbohydrate-Active enZymes (CAZymes). CAZymes have been classified in sequenced-based families as glycoside hydrolases (GH), glycosyltransferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE) and auxiliary activities (AA) *[89]*.

Cellulose degrading enzymes

Cellulase (EC 3.2.1.4, family GH5, 7 and 12) is needed for the enzymatic degradation of cellulose. It catalyzes the endohydrolysis of β-(1,4)-D-glucosidic linkages in cellulose. Further degradation into cellobiose and glucose can be obtained by the respective action of the enzymes cellobiohydrolase (EC 3.2.1.91, family GH6 and 7) and β-glucosidase (EC 3.2.1.21, family GH1 and 3) *[90]*. Recently, a new class of enzymes that catalyze oxidative cleavage of cellulose has been discovered. These enzymes promote the efficiency of classical hydrolytic cellulases by acting on the surfaces of the insoluble substrate, where they introduce chain breaks in the polysaccharide chains, without the need of first "extracting" these chains from their crystalline matrix. After initially being classified as CBM33 family and GH61 members *[91, 92]*, it was recently shown that this new class of enzymes are in fact lytic polysaccharide monooxygenases (LPMO). Therefore, CAZy has been revised and CBM33 and GH61 families are now named LPMO and classified as families AA9 and AA10 *[93]*.

Pectin degrading enzymes

As already illustrated in Figure 1.3, the structure of sugar beet pectin is complex and many different enzymes, belonging to the different GH-, PL, or CE-families, are needed to release all of its constituents. An overview of sugar beet pectin degrading enzymes is presented in Table 1.2, while Table 1.3 provides an overview of the reactions catalyzed by the different pectinolytic enzymes.

HG degrading enzymes

HG is the 'simplest' structural element of pectin. Nevertheless, several enzymes are needed for its complete degradation (Figure 1.7). HG can be de-esterified by pectin methyl esterase (PME) and pectin acetyl esterase (PAE). The main chain of HG can be cleaved by endo- and exopolygalacturonases (PGA, PGX). Lyases are also involved in the degradation of pectin by cleaving the backbone by β-elimination. The activity of these enzymes depends on the degree of methylesterification. Pectin lyases (PEL) prefer a highly esterified HG in contrast to pectate lyases (PLY) *[42]*. The mode of action of the enzymes may differ, depending on the enzyme source. For example, the mechanism of demethylesterification of HG by PME's can be in a blockwise, random or multiple-attack manner *[94]*.

Table 1.2. Overview of Carbohydrate-Active Enzymes (CAZymes) involved in the degradation of sugar beet pectin *[42]*.

Enzyme name	Abbreviation	Family	EC-number
Endo-polygalacturonase	PGA	GH28	EC 3.2.1.15
Exo-polygalacturonase	PGX	GH28	EC 3.2.1.67
Pectin lyase	PEL	PL1	EC 4.2.2.10
Pectate lyase	PLY	PL1,3,9	EC 4.2.2.2
Pectin methylesterase	PME	CE8	EC 3.1.1.11
Pectin acetylesterase	PAE	CE12	EC 3.1.1.6
Rhamnogalacturonan hydrolase	RGH	GH28	EC 3.1.1.171
Unsaturated rhamnogalacturonyl hydrolase	URH	GH105	EC 3.2.1.172
Rhamnogalacturonan galacturonohydrolase	RGX	GH28	EC 3.2.1.173
Rhamnogalacturonan rhamnohydrolase	RHA	GH78	EC 3.2.1.174
Rhamnogalacturonan lyase	RGL	PL4,11	EC 4.2.2.23/24
Rhamnogalacturonan acetylesterase	RGAE	CE12	EC 3.1.1.86
Endo-arabinanase	ABN	GH43	EC 3.2.1.99
Exo-arabinanase	ABX	GH93	EC 3.2.1.-
Arabinofuranosidase	ABF	GH43,51,54	EC 3.2.1.55
Endo-galactanase	GAL	GH53	EC 3.2.1.89
β-Galactosidase	LAC	GH2,35	EC 3.2.1.23
Ferulic acid esterase	FAE	CE1	EC 3.1.1.73

Table 1.3. Catalyzed reactions by the different enzymes involved in pectin degradation.

Enzyme abbreviation	Reaction	Reference(s)
PGA	Hydrolysis of (1,4)-α-D-galactosiduronic linkages in pectate and other galacturonan backbones	*[95]*
PGX	Release of terminal galacturonosyl residues from the non-reducing end of HG or HG-oligosaccharides	*[96]*
PEL	Eliminative cleavage of (1,4)-α-D-galacturonan (preferentially methyl esterified) to give oligosaccharides with 4-deoxy-(6-*O*-methyl-)α-D-galact-4-enuronosyl groups at their non-reducing ends	*[97, 98]*
PLX	Eliminative cleavage of (1,4)-α-D-galacturonan to give oligosaccharides with 4-deoxy-α-D-galact-4-enuronosyl groups at their non-reducing ends	*[99]*
PME	Removal of methyl ester at the C6 position of galacturonic acid residues	*[100, 101]*
PAE	Removal of acetyl groups present in the HG region of pectin	*[102]*
RGH	Hydrolysis of α-D-GalA-(1,2)-α-L-Rha glycosidic linkage in the RG I backbone with initial inversion of anomeric configuration releasing oligosaccharides with β-D-GalA at the reducing end	*[103, 104]*
URH	Release of unsaturated galacturonic acid from the non-reducing end of unsaturated RG I or RG I oligosaccharides	*[105, 106]*
RGX	Release of galacturonosyl residues from the non-reducing end of RG or RG-oligosaccharides	*[107, 108]*
RHA	Release of rhamnosyl residues from the non-reducing end of RG-oligosaccharides	*[108-110]*
RGL	Cleavage of α-L-Rha*p*-(1,4)-α-D-Gal*p*A linkages in RG I by β-elimination	*[103, 111]*
RGAE	Removal of 2-*O*-acetyl- or 3-*O*-acetyl groups from α-D-galacturonic acid in RG I	*[112]*
ABN	Hydrolysis of (1,5)-α-arabinofuranosidic linkages in (1,5)-α-L-arabinans	*[113, 114]*
ABX	Release of arabinose, arabinobiose or arabinotriose from the non-reducing end of the α-(1,5)-linked arabinan backbone	*[115]*
ABF	Release of terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides	*[116]*
GAL	Hydrolysis of β-(1,3)- and β-(1,4)-linkages in galactan and galacto-oligosaccharides	*[117, 118]*
LAC	Release of terminal non-reducing β-D-galactose residues in β-D-galactosides	*[119]*
FAE	Release of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar	*[120]*

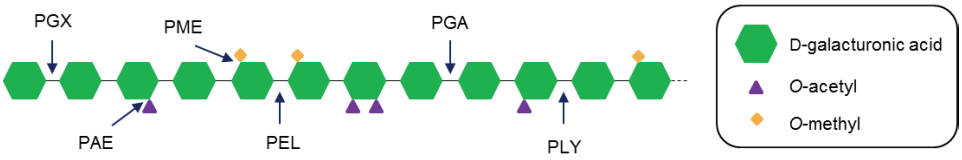

Figure 1.7. Schematic structure of homogalacturonan and enzymes needed for its degradation. Adapted from *[6, 42]*. Annotation: PGA, endo-polygalacturonase; PGX, exo-polygalacturonase; PME, pectin methylesterase; PAE, pectin acetylesterase; PEL, pectin lyase; PLY, pectate lyase.

RG I degrading enzymes

Due to its complexity, various specifically acting enzymes are needed for the complete degradation of RG I (Figure 1.8). Two types of endo-enzymes are known to catalyze bond cleavage within the RG I backbone: Rhamnogalacturonan hydrolase (RGH) and rhamnogalacturonan lyase (RGL) *[103]*. In addition to these endo-enzymes, three exo-acting enzymes have also been characterized. Unsaturated rhamnogalacturonyl hydrolase (URH) acts specifically on unsaturated rhamnogalacturonan (structure resulting from the action of RGL on RG I) to release the terminal unsaturated galacturonic acid *[106]*. Rhamnogalacturonan rhamnohydrolase (RHA) removes terminal rhamnosyl residues, whereas rhamnogalacturonan galacturonohydrolase (RGX) removes terminal galacturonosyl residues from the non-reducing end of RG I or RG I oligosaccharides *[108]*. Rhamnogalacturonan acetylesterase (RGAE) de-acetylates the RG I backbone by hydrolytic cleavage of the acetyl groups at the *O*-2 or *O*-3-positions of galacturonic acid residues *[112]*. For the degradation of the galactan side chains of RG I, endo-galactanase (GAL) and β-galactosidase (LAC) are needed. For the degradation of the heavily branched sugar beet arabinan side chains, a group of arabinohydrolases is required *[113]*. Endo-arabinanase (ABN) hydrolyzes the α-(1,5)-linkages in the unsubstituted regions of the arabinan backbone. Exo-arabinanase (ABX) releases arabinose, arabinobiose and arabinotriose from the non-reducing end of the α-(1,5)-linked arabinan backbone. Arabinofuranosidase (ABF) is able to release monomeric arabinose from all non-reducing ends of arabinan or arabino-oligosaccharides. For the removal of feruloyl groups from the galactan or arabinan side chains, the enzyme ferulic acid esterase (FAE) is needed *[120]*.

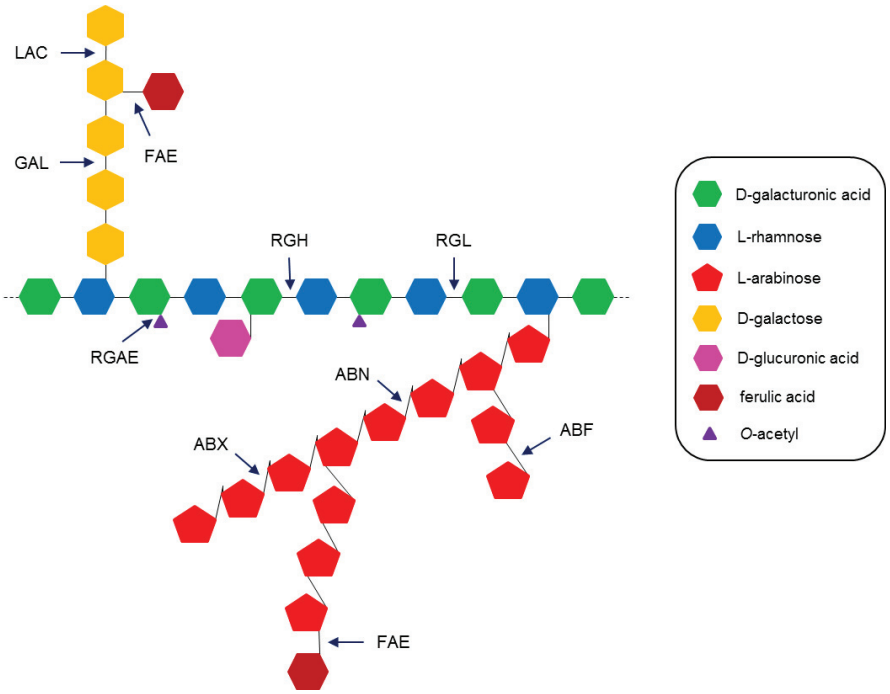

Figure 1.8. Schematic structure of sugar beet rhamnogalacturonan I and enzymes needed for its degradation. Adapted from *[6, 42]*. Annotation: RGH, rhamnogalacturonan hydrolase; RGL, rhamnogalacturonan lyase; PME, pectin methylesterase; RGAE, rhamnogalacturonan acetylesterase; ABN, endo-arabinanase; ABX, exo-arabinanase; ABF, arabinofuranosidase; GAL, endo-galactanase; LAC, β-galactosidase; FAE, ferulic acid esterase.

RG II degrading enzymes

Thus far, enzymes specific for degrading RG II have not yet been isolated *[121]*. However, it has been envisioned that some of the HG or RG I degrading enzymes might also act on RG II *[122]*.

1.4.3 Efficiency of enzymatic saccharification

Several studies on the saccharification of sugar beet pulp have been described, although most studies were conducted on small laboratory (gram) scale. Enzymatic pectin hydrolysis up to 95% monomers has been demonstrated *[123, 124]*, but this involved long incubation times (120 hours) and a low sugar beet pulp dry matter content (2.5-4%). Furthermore, over 60% of the cellulose was degraded which is undesired if simultaneous production of cellulose fibers and pectin derived

monosaccharides from sugar beet pulp is aimed for. Pectin hydrolysis with a monosaccharide yield of 70-80% and almost no cellulose degradation was obtained with incubation times of 24-48 hours, but required the use of a mix of many different commercial enzyme preparations and a sugar beet pulp dry matter content of maximally 1% *[4]*. Another study showed a yield of 71% galacturonic acid and 67% arabinose, using a single commercial enzyme preparation, an incubation time of 48 hours and a 3% sugar beet pulp dosage *[125]*. Within this study, it was also shown that the end-products of enzymatic saccharification were composed of mixtures of monosaccharides and several complex enzyme resistant pectin derived structures, mainly rhamnogalacturonan-derived oligosaccharides.
To develop an economically feasible large-scale biorefinery process for sugar beet pulp, the efficient, specific and cost-effective enzymatic degradation of sugar beet pectin is of high importance.

1.5 Characterization of plant cell wall derived oligosaccharides

Enzymatic or thermo-chemical treatment of plant cell wall containing biomass will degrade the cell wall polysaccharide structure. Uncomplete degradation results in mixtures of mono- and oligosaccharides. To evaluate the effectiveness of the selected technology, it is important to obtain detailed knowledge on the structure of the oligosaccharides that are formed. Nowadays, many different analytical tools are available for the characterization of oligosaccharides (Table 1.4).

1.5.1 Analysis of the gross composition of mixtures of oligosaccharides

Constituent monosaccharide composition
The constituent monosaccharide composition of oligosaccharides can be analyzed after hydrolysis or methanolysis of the oligosaccharides. Commonly used hydrolytic agents for cleavage of glycosidic bonds are sulfuric acid or trifluoroacetic acid (TFA) *[126, 127]*. The susceptibility of different glycosidic linkages varies. Uronic acids are often involved in rather acid-resistent glycosidic linkages, which are not easily/completely degraded using acid hydrolysis *[127, 128]*. Furthermore, decomposition of liberated monosaccharides may occur. Uronic acids can undergo decarboxylation reactions when harsh acid hydrolysis conditions (using concentrated acids and high temperatures) are applied *[129]*.

Table 1.4. Overview of analytical methods for characterization of plant cell wall derived oligosaccharides, including possible analytical restrictions.

Methods	Reference(s)
Analysis of gross composition of mixtures of oligosaccharides	
Constituent monosaccharide composition (after hydrolysis or methanolysis)	
▪ Determination as alditol acetates (using GC-FID/MS) *(acidic monosaccharides are not included in analysis)*	*[130, 131]*
▪ Determination as trimethylsilyl (TMS) derivatives (using GC-MS) *(both neutral and acidic monosaccharides are included in analysis)*	*[132]*
▪ Determination as underivatized monosaccharides (using HPAEC-PAD)	*[127]*
▪ Determination of total uronic acid content (using *m*-hydroxydiphenyl assay)	*[133, 134]*
Determination of the degree of acetylation and methyl esterification (using HPLC)	*[135, 136]*
Determination of the degree of feruloylation (using HPLC)	*[60, 137]*
Determination of molecular mass distribution (using HPSEC-RI/UV)	*[3]*
Determination of molecular mass distribution (using HPSEC-MALLS-RI)	*[138]*
Determination of molecular masses (*m/z*) present (using MALDI-TOF MS)	*[139, 140]*
Determination of glycosidic linkage types present (using GC-FID/MS)	*[141, 142]*
Separation and characterization of individual oligosaccharides in complex mixtures	
Using HPAEC-PAD *(loss of alkali-labile esterified substituents, no mass detection)*	*[143]*
Using HPAEC-MS *(loss of alkali-labile substituents and desalting of eluent required for coupling with MS)*	*[144, 145]*
Using RP-HPLC-ELSD-MS *(minimal retention and separation of acidic oligosaccharides)*	*[146]*
Using IP-RP-HPLC-MS *(removal of ion-pairing reagents required for coupling with MS)*	*[147]*
Using CE-LIF *(co-elution of acidic oligosaccharides, no mass detection)*	*[148]*
Using CE-MS *(separation based on total negative charge)*	*[149]*
Using PGC-HPLC-ELSD-MS *(co-elution of acidic oligosaccharides with equal charge)*	*[150, 151]*
Using HILIC-MS *(little information available about separation potential for acidic plant derived oligosaccharides)*	*[152, 153]*
Determination of oligosaccharide sequence and branching configuration	
Using NMR *(purified samples required, no analysis of complex mixtures possible)*	*[154]*
Using MS^n *(difficult to distinguish glycosyl residues having the same mass, e.g. arabinose/xylose)*	*[155]*
Using ion-mobility MS *(coupling with liquid chromatography, required for analysis of complex mixtures, is difficult)*	*[156, 157]*

Instead of acid hydrolysis, acid methanolysis has been described as a rapid and convenient technique for analysis of pectins and hemicelluloses. Methanolysis results in high yields of both neutral sugar and uronic acids *[129]*. Acid methanolysis followed by TFA hydrolysis has been reported to be able to completely liberate monosaccharides from pectic material with minimal concomitant degradation of the monosaccharides released *[127]*. The neutral monosaccharides released upon hydrolysis or methanolysis can either be analyzed as alditol-acetates using gas chromatography with flame ionization detection (GC-FID) *[130]* or with mass spectrometry detection (GC-MS) *[131]*, as trimethylsilyl methyl glycoside derivatives using GC-MS *[132]* or as underivatized monosaccharides using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) *[127]*. Quantification of the uronic acid content is still often performed using the (automated) colorimetric *m*-hydroxydiphenyl assay *[133, 134]*.
These methods provide information on the average monosaccharide composition of oligosaccharides. However, information on the linkages present, the distribution of the various monosaccharides and the presence of non-sugar substituents within the oligosaccharides is not obtained.

Determination of glycosidic linkage types
Glycosidic linkage type analysis of the sugar residues present in oligosaccharides has been described using GC-FID/MS of the monosaccharides after permethylation, hydrolysis and subsequent conversion into partial alditol acetates *[141, 142]*. This provides information on the types of glycosidic linkages present in a mixture, but does not resolve the linkages present within individual molecules or the sequence of the monosaccharides within the oligosaccharide.

Analysis of non-sugar substituents
The esterification of galacturonic acid residues with methanol and/or acetic acid is an important structural feature of pectic substances *[158]*. The degree of methyl esterification (DM) corresponds to the percentage of carboxyl groups esterified with methanol. The degree of acetylation (DA) is defined as the percentage of galacturonosyl residues substituted with an acetyl group *[37]*. The DA can be larger than 100% as galacturonosyl residues can carry two acetyl groups. Several methods have been developed to quantify the number of methyl esters and acetyl groups in pectic substances. These methods usually involve saponification followed by methanol and acetic acid analysis and quantification using high performance liquid chromatography (HPLC) with refractive index (RI) detection *[135, 136]*.

Besides the esterification of galacturonosyl residues, arabinosyl and galactosyl residues may be feruloylated. These feruloyl groups can be released by saponification and subsequently quantified using either a spectrophotometric determination *[137]* or using reversed phase HPLC with diode array detection (RP-HPLC-DAD) *[60]*. Recently, a quantitative ^{1}H NMR-based method for the rapid determination of acetyl and feruloyl esters in plant polysaccharides has been described *[159]*.

Determination of molecular masses
To monitor the degradation of polysaccharides into oligo- and monosaccharides, useful information on the molecular mass distribution may be obtained using high performance size exclusion chromatography with refractive index and ultraviolet detection (HPSEC-RI/UV) *[3]* or HPSEC with multi-angle laser light scattering (MALLS) and RI-detection *[138]*. In addition to this, matrix-assisted laser desorption/ionisation−time-of-flight mass spectrometry (MALDI-TOF MS) is a valuable analytical tool for the rapid determination of the mass-to-charge ratio (*m/z*) of individual neutral and acidic oligosaccharides *[139, 140]*. However, MALDI-TOF MS cannot directly distinguish isomeric oligosaccharides which differ in anomerity or branching configuration.

1.5.2 Analysis of complex mixtures of oligosaccharides

Nowadays, many different analytical techniques are available for the analysis of complex mixtures of oligosaccharides (Table 1.4).
HPAEC-PAD is a commonly used technique for the analysis of oligosaccharides *[143]*. Unfortunately, the high pH eluent used for separation and detection results in saponification of methyl esters and acetyl groups present on the oligosaccharides, thereby losing important structural information *[27]*. The coupling of HPAEC with MS has been demonstrated for analysis of oligosaccharides not carrying alkali-labile substituents. However, the high salt content used within the eluent requires desalting to make HPAEC compatible with MS, while only limited levels of salts can be removed *[144]*,
Reversed phase (RP)-HPLC coupled with evaporative light scattering detection (ELSD) and MS has been used for separation and characterization of neutral xylo-oligosaccharides, thereby leaving *O*-acetyl substituents intact *[146]*. It is known, however, that acidic oligosaccharides show minimal retention on RP-columns due to their high polarity *[160]*. Ion-pairing chromatography is able to overcome this problem for a large part, but on-line removal of the ion-pairing reagents prior to MS is required to prevent contamination of the electrospray ionization (ESI) interface and signal suppression of the analytes of interest *[161]*.

Oligosaccharide analysis using capillary electrophoresis (CE) with laser-induced fluorescence (LIF) and MS has also been described *[148, 149]*. Fast separations of different plant cell wall derived oligosaccharides have been obtained, although drawbacks have also been observed with this technique. Labelling of oligosaccharides with a fluorescent chromophore at the reducing end is required, which excludes non-reducing oligosaccharides from analysis. Additionally, acidic oligosaccharides migrate too fast due to the additional negative charges, making them difficult to separate *[149]*.

Porous-graphitized carbon (PGC) HPLC-ELSD-MS has been described as a versatile analytical technique for the separation and characterization of different oligosaccharides *[151, 162, 163]*. Promising results have been obtained for neutral and acidic cell wall derived oligosaccharides *[150]*. Using this technique, structural information regarding acetyl groups and methyl esters is retained, making this technique very useful for analysis of pectin derived oligosaccharides. However, the elution behavior strongly depends on the net charge of the molecules. This leads to coelution of oligosaccharides with an equal number of free acid groups, but a different degree of polymerization (DP), as is shown in Figure 1.9. This coelution can limit the use of PGC HPLC if detailed information is required on the methyl ester distribution.

In recent years, hydrophilic interaction chromatography (HILIC) has been described as an alternative method of separation of highly polar compounds. The term HILIC was introduced to distinguish this chromatographic mode from normal and reverse phase chromatography *[164]*. It uses a hydrophilic stationary phase (base material of silica or polymer particles, modified with different types of polar functionalities such as amide, amino, diol or cyano) in combination with a mostly organic mobile phase. Elution of analytes from the column is usually performed by increasing the concentration of water in the mobile phase. Its separation efficiency has been demonstrated for different mono-, di- and trisaccharides, a range of maltooligosaccharides, labelled xyloglucan and xylan-derived oligosaccharides *[152, 153, 165-170]*. Its use has also been demonstrated for structural glycomics *[171]*. However, there is not much information available yet on its ability to separate other plant derived uronic acid containing oligosaccharides. The high fraction of organic solvent used in HILIC mobile phases (usually acetonitrile) provides a higher sensitivity in ESI-MS, due to efficient droplet formation and desolvation within the MS source and faster separations due to the lower viscosity, compared to the highly aqueous RP-mobile phases *[172, 173]*. However, solubilization of large oligosaccharides in the high organic solvent concentrations may be difficult *[171]*.

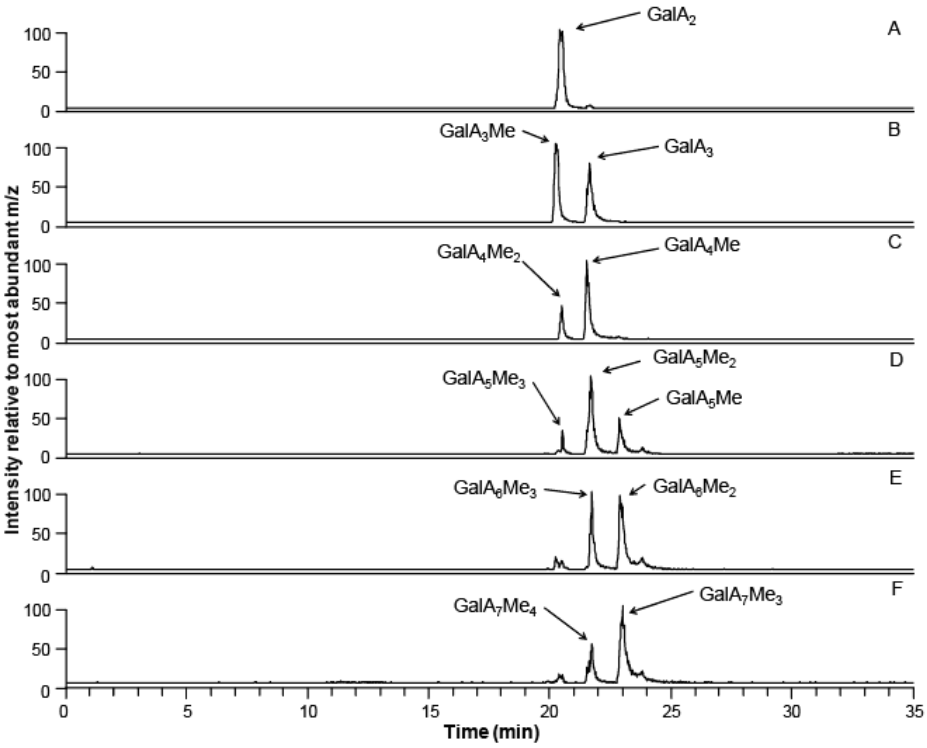

Figure 1.9. Porous-graphitized carbon (PGC) HPLC-MS elution profile of partly methylesterified galacturono-oligosaccharides derived from a DM30-pectin after treatment with endopolygalacturonase: Selected ion chromatogram (SIC) of (A) $GalA_2$; (B) $GalA_3$ and $GalA_3Me$; (C) $GalA_4Me$ and $GalA_4Me_2$; (D) $GalA_5Me$, $GalA_5Me_2$ and $GalA_5Me_3$; (E) $GalA_6Me_2$ and $GalA_6Me_3$; (F) $GalA_7Me_3$ and $GalA_7Me_4$. GalA, galacturonic acid; Me, methyl ester. Adapted from *[150]*.

1.5.3 Detailed characterization of oligosaccharides

Nuclear magnetic resonance spectroscopy (NMR) enables detailed elucidation of the structural features of oligosaccharides, such as sequence, linkage and branching. The structural assignment is based on analysis of several spectroscopic parameters for each magnetically active nucleus (e.g., ^{1}H or ^{13}C) in the oligosaccharide *[154]*. The main drawbacks of NMR are sample purity and concentration requirements *[155]*. The one- and two-dimensional NMR techniques commonly used for determining a complete primary structure require time-consuming purification steps to obtain sufficient material of the oligosaccharide of

interest *[16]*. Besides NMR, mass spectrometers with soft ionization techniques (e.g., electrospray ionization (ESI)) and capability to carry out multiple stages of isolation and dissociation of ions (so called MS^n) allow detailed structural characterization of oligosaccharides. Glycosyl sequence information is obtained by analyzing fragment ions generated in the MS source. The advantage of MS^n is that parent ions with a specific mass to charge (*m/z*) ratio can be selected for fragmentation to produce a daughter ion spectrum. Due to well-described fragmentation pathways (as illustrated in Figure 1.10), the glycosyl sequence can be derived from the daughter ion spectra *[174]*. However, unambiguous determination of the glycosyl sequence is not always possible using MS^n, due to equal masses of glycosyl residues (e.g., glucose and galactose), molecular rearrangements or the generation of inner fragments by multiple cleavage processes. The likelihood that specific, well-characterized fragmentation reactions will occur can be facilitated by converting the oligosaccharides to their per-*O*-acetylated or per-*O*-methylated derivatives, which can also reduce the complexity of the daughter ion spectra *[16]*. Reduction of the complexity of MS-spectra can also be achieved by labeling of the reducing end of oligosaccharides, e.g. with 2-aminobenzamide or 3-aminoquinoline *[175]*. In addition to these techniques, ion mobility-MS has been demonstrated to be valuable in the evaluation of isomeric oligosaccharides that yield identical sets of product ions in MS^n experiments by being capable of separating different structural oligomers with identical mass-to-charge (*m/z*) ratios *[156]*. An important advantage of MS-techniques is their compatibility with liquid chromatography, which enables separation of complex mixtures prior to MS without the need for purification.

As described above, several analytical techniques are already available to separate and characterize plant cell wall derived oligosaccharides, but each technique has its own limitations for simultaneous analysis of a broad range of oligosaccharides. The main limitation observed for the analysis of pectin digests is the lack of an analytical technique that is able to separate and characterize both neutral and acidic oligosaccharides, thereby separating acidic oligosaccharides having the same total negative charge, yet different degree of polymerization or degree of substitution (e.g. as shown in Figure 1.9). During analysis, important structural features of the oligosaccharides, such as acetyl or methyl ester groups, must be retained. In addition, an analytical tool is lacking for the separation and characterization of complex mixtures of isomeric pectin derived oligosaccharides.

Given the above, the analysis of complex mixtures of plant cell wall derived oligosaccharides still requires a combination of time-consuming fractionation and/or use of different analytical techniques. Therefore, there is a need to develop a versatile technique for unambiguous identification of a broad range of neutral and acidic plant cell wall derived oligosaccharides.

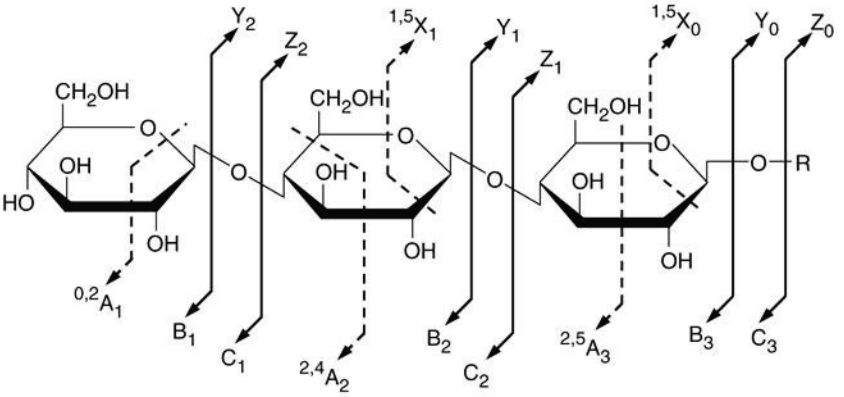

Figure 1.10. Carbohydrate fragmentation types and their nomenclature as described by Domon and Costello *[174]*. In this nomenclature, ions retaining the charge on the non-reducing terminus are named A, B and C, and the ions retaining charge on the reducing terminus are X, Y and Z. A and X correspond to cross-ring cleavages, whereas B, C, Y and Z correspond to glycosidic cleavages. Subscript numbers denote the cleavage position, starting at the reducing terminus for the X, Y and Z ions, and at the non-reducing terminus for the others. In the case of ring cleavages, superscript numbers are given to show the cleaved bonds *[176]*.

1.6 Aim and outline of the thesis

The aim of this thesis was to develop an additional versatile analytical method to characterize complex mixtures of sugar beet pulp derived oligosaccharides, to be able to monitor and optimize the enzymatic saccharification of sugar beet pulp. To this end, novel LC-MS approaches were developed. Furthermore, the *in vitro* fermentation characteristics of sugar beet pulp derived oligosaccharides were studied to investigate their potential as functional food or feed ingredient.

In Chapter 1, the background of the project is presented. The composition of sugar beet pulp, its valorization opportunities and its saccharification are discussed and a review on the different analytical tools that are available for the characterization of plant cell wall derived oligosaccharides is provided.

Analysis of complex mixtures of plant cell wall derived oligosaccharides is challenging and multiple analytical techniques are often required for separation and characterization of these mixtures. Therefore, a HILIC-ELSD-MS method was developed as a versatile and additional technique for the analysis of a wide range of neutral and acidic plant cell wall derived oligosaccharides (Chapter 2). This

method was used as an analytical tool to monitor and optimize the enzymatic saccharification of sugar beet pulp. By using this method, the recalcitrant oligosaccharides that were obtained after enzymatic saccharification were characterized. This enabled recognition of enzymes needed for further degradation (Chapter 3). The possible utilization of the oligosaccharides that were obtained after enzymatic saccharification of sugar beet pulp as functional food or feed ingredient was evaluated by studying their *in vitro* fermentation characteristics using fecal inocula from humans and pigs. In addition, the fate of the different types of oligosaccharides and the changes in human fecal bacterial populations during *in vitro* fermentation were described (Chapter 4). In Chapter 5, the coupling of HILIC with ion-mobility-MS is introduced as an additional analytical technique for the separation and characterization of complex mixtures of isomeric pectin derived oligosaccharides.
Chapter 6 discusses the main results and insights obtained during the study and highlights the relevance of this thesis for other fields of research. Future perspectives on the use of analytical techniques to study plant cell wall derived oligosaccharides are discussed.

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Chapter 2

Characterizing plant cell wall derived oligosaccharides using hydrophilic interaction chromatography with mass spectrometry detection

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Abstract

Analysis of complex mixtures of plant cell wall derived oligosaccharides is still challenging and multiple analytical techniques are often required for separation and characterization of these mixtures. In this work it is demonstrated that hydrophilic interaction chromatography coupled with evaporative light scattering and mass spectrometry detection (HILIC-ELSD-MS^n) is a valuable tool for identification of a wide range of neutral and acidic plant cell wall derived oligosaccharides. The separation potential for acidic oligosaccharides observed with HILIC is much better compared to other existing techniques, like capillary electrophoresis, reversed phase and porous-graphitized carbon chromatography. Important structural information, such as presence of methyl esters and acetyl groups, is retained during analysis. Separation of acidic oligosaccharides with equal charge yet with different degrees of polymerization can be obtained. The efficient coupling of HILIC with ELSD and MS^n-detection enables characterization and quantification of many different oligosaccharide structures present in complex mixtures. This makes HILIC-ELSD-MS^n a versatile and powerful additional technique in plant cell wall analysis.

2.1 Introduction

Several pretreatment technologies can be applied to release useful components from plant cell wall containing biomass (e.g. enzymatic treatment, physico-chemical techniques). Enzymatic processes will degrade the cell wall polysaccharide structure resulting in complex mixtures of mono-, oligo- and polysaccharides. To evaluate the effectiveness of the selected technology and the application properties of the product it is important to obtain detailed knowledge on the structure of the oligosaccharide fragments that are formed. Several analytical techniques are already available to separate and characterize these structural elements, but each technique has its own limitations for simultaneous analysis of a broad range of cell wall derived oligosaccharides.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is frequently used for mono- and oligosaccharide analysis *[1]*. Unfortunately, the high pH eluent used for separation and detection results in saponification of methyl esters and acetyl groups from the analytes *[2]*. The high salt content used within the eluent requires desalting before HPAEC can be coupled with mass spectrometry *[3]*, while only limited levels of salts can be removed.

Reversed phase high performance liquid chromatography coupled with evaporative light scattering and mass spectrometry detection (RP-HPLC-ELSD-MS) has proven to be useful for separation and characterization of neutral xylo-oligosaccharides while leaving *O*-acetyl substituents intact *[4]*. It is known, however, that acidic oligosaccharides show minimal retention on RP-columns due to their high polarity *[5]*. Ion-pairing chromatography is able to overcome this problem for a large part, but on-line removal of the ion-pairing reagents prior to MS is required to prevent contamination of the electrospray ionization (ESI) interface and signal suppression of the analytes of interest *[6]*.

Oligosaccharide analysis using capillary electrophoresis (CE) with laser-induced fluorescence (LIF) and mass spectrometry detection has been described recently *[7, 8]*. Fast separations of different plant cell wall derived oligosaccharides have been obtained. Some drawbacks have also been observed with this technique. Labelling of oligosaccharides with a fluorescent chromophore at the reducing end is required, which excludes non-reducing sugars from analysis. Additionally, acidic oligosaccharides migrate too fast due to the additional negative charges, making them difficult to separate *[8]*.

It has been demonstrated that porous-graphitized carbon (PGC) HPLC-ELSD-MS can be widely used for separation and characterization of many different oligosaccharides *[9-11]*. Promising results have been obtained recently for neutral and acidic oligosaccharides derived from cell wall polysaccharides *[12]*. This

technique enables the characterization of oligosaccharides carrying e.g. acetyl groups and methyl esters. Neutral oligosaccharides are separated based on their size as well as on their type of linkage and resulting 3D-structure. Charged oligomers, such as α-(1,4)-galacturonic acid oligosaccharides, are strongly retained and are eluted only after addition of an acidic modifier. However, the elution behavior depends on the net charge of the molecules. This leads to coelution of oligosaccharides with an equal number of free acid groups but a different degree of polymerization (DP) *[12]*. This coelution can be disadvantageous if detailed information is required on the composition of the structural elements in pectin digests.

Matrix-assisted laser desorption/ionisation – time-of-flight mass spectrometry (MALDI-TOF MS) is a valuable additional analytical tool for the determination of the molecular masses of neutral and acidic oligosaccharides *[13, 14]*. However, MALDI-TOF MS cannot directly distinguish anomerity or branching configuration of oligosaccharides. More detailed structural information can be obtained by observing the fragmentation behaviour using electrospray ionization ion trap MS where multiple stages of isolation and dissociation (MS^n) are possible. In addition to these techniques ion mobility-MS has demonstrated to be valuable in the evaluation of isomeric oligosaccharides that yield identical sets of product ions in MS^n experiments by being capable of separating different structural oligomers with identical mass-to-charge (*m/z*) ratios *[15]*.

Hydrophilic interaction chromatography (HILIC) is an alternative method of separation of highly polar compounds. HILIC was introduced to distinguish this chromatographic mode from normal and reverse phase chromatography *[16]*. It uses a hydrophilic stationary phase (base material of silica or polymer particles, modified with different types of polar functionalities such as amide, amino, diol or cyano) in combination with a mostly organic mobile phase and elution is usually performed by increasing the water concentration. Its separation efficiency has been demonstrated for different mono-, di- and trisaccharides, a range of maltooligosaccharides, labelled xyloglucans and xylan-derived oligosaccharides *[17-24]*. Its use has also been demonstrated for structural glycomics *[25]*. However, there is not much information available yet on its ability to separate other plant derived oligosaccharides, e.g. uronic acid containing oligosaccharides. The high fraction of organic solvent used in HILIC mobile phases (usually acetonitrile) provides higher sensitivity in ESI-MS due to efficient droplet formation and desolvation within the MS source and faster separations due to the lower viscosity compared to the highly aqueous RP-mobile phases *[26, 27]*.

Analysis of complex mixtures of plant cell wall derived oligosaccharides requires a combination of several of the above mentioned analytical techniques for effective separation and characterization. In this chapter, it is described how HILIC in

combination with ELS- and MS-detection fulfills the need for a rapid and single technique for identification of a broad range of neutral and acidic cell wall derived oligosaccharides.

2.2 Experimental

2.2.1 Materials

All chemicals used were at least of analytical grade. Water was purified by a MilliQ®-system (Millipore, Billerica, MA, USA). Acetonitrile (ACN) was of ULC/MS grade and was purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid and ammonium formate were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate and ammonium hydroxide were purchased from Merck (Darmstadt, Germany).
Samples of α-(1,5)-arabinose, α-(1,4)-glucose, β-(1,4)-glucose, β-(1,4)-mannose, β-(1,4)-xylose, β-(1-4)-galactose, α-(1,4)-galacturonic acid and xylogalacturonan oligosaccharides were obtained as described by Westphal *et al. [12]*. Partially methyl esterified α-(1,4)-galacturonic acid oligosaccharides were obtained after digestion of a 30%-methyl esterified pectin (DM30) with endo-polygalacturonase from *Aspergillus niger*. A mixture of partially saturated, unsaturated and methyl esterified α-(1,4)-galacturonic acid oligosaccharides was obtained after digestion of a 56%-methyl esterified pectin (DM56) with endo-polygalacturonase and pectin lyase (both from *Aspergillus niger*). Partially methyl esterified and acetylated galacturonic acid oligosaccharides were obtained after modification and digestion of sugar beet pectin with fungal pectin esterase as described earlier *[28]*. Sugar beet pulp hydrolysates were obtained from Cosun Food Technology Centre (Roosendaal, The Netherlands). The hydrolysates were prepared by digestion of sugar beet pulp with a mixture of commercially available pectinases.
All samples were diluted to 1 mg/mL. Samples were diluted with 50:50 (v/v) ACN/water and centrifuged as a compromise between analyte solubility and optimal peak shape and preventing polymeric material to be injected onto the column.

2.2.2 Hydrophilic interaction liquid chromatography with evaporative light scattering and mass spectrometry detection

UPLC-ELSD-MS^n method development was carried out on an Accela UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled to an evaporative light scattering detector (ELSD 85, Sedere, Alfortville Cedex, France) and an ESI-MS^n-detector (LTQ XL MS, ion trap, Thermo Scientific, San Jose, CA, USA).

Chromatographic separation was performed on an Acquity UPLC BEH Amide column (1.7 µm, 2.1 mm x 150 mm) in combination with an Acquity UPLC BEH Amide VanGuard precolumn (1.7 µm, 2.1 mm x 5 mm; Waters Corporation, Milford, MA, USA). Elution was performed at a flow rate of 600 µL/min and a column oven temperature of 35 °C. The injection volume was set to 10 µL. In order to prevent distorted or split peaks in the chromatograms a strong and weak needle wash procedure was applied. Strong and weak needle wash solvents contained 20:80 (v/v) ACN/water and 75:25 (v/v) ACN/water, respectively. Three double stage mobile phases have been evaluated, varying in pH and buffer composition. Mobile phase at aqueous pH 3 was composed of (A) 80:20 (v/v) ACN/water and (B) 20:80 (v/v) ACN/water. To both eluents 10 mM ammonium formate and 0.2% (v/v) formic acid were added. Mobile phase at aqueous pH 6.8 was composed of (A) 80:20 (v/v) ACN/water and (B) 20:80 (v/v) ACN/water, with 10 mM ammonium acetate added to both eluents. Mobile phase at aqueous pH 10 was composed of (A) 80:20 (v/v) ACN/water and (B) 20:80 (v/v) ACN/water, with 0.1% (v/v) ammonium hydroxide added to both eluents. Optimal run time for separation of various oligosaccharides was 40 minutes using the following gradient elution profile: 0-1 min, isocratic 100% A; 1-31 min, linear from 30 to 80% (v/v) B; followed by column re-equilibration: 31-32 min linear from 20% A to 100% A; 32-40 min, isocratic 100% A. Gradient steepness was adjusted for samples containing components with particularly high or low retention in order to obtain improved separation. The Acquity BEH Amide column was coupled to a 1:1-splitter (Accurate, Dionex Corporation, Sunnyvale, CA, USA) directing the eluent both to the ELSD and to the ESI-MS^n-detector. The ELSD micro flow nebulizer (Sedere) had a gas pressure of 3.5 bar and a gas flow of 1.75 L/min. The drift tube temperature of the ELSD was set to 50 °C and the gain to 12. MS-detection was performed in negative mode with the ion source voltage set to −4.5 kV, capillary voltage −5 V, capillary temperature 350 °C, sheath gas 40 (arbitrary units), auxiliary gas 5 (arbitrary units) and auto-tuned on tetragalacturonic acid (*m/z* 721). Mass spectra were acquired over the scan range *m/z* 150-2000. MS^n-collection parameters included normalized collision energy 30 (arbitrary units), activation Q 0.25 (arbitrary units), activation time 30 ms and isolation width 2 m/z. Data were processed using XCalibur software (Thermo Scientific).

2.3 Results and discussion

To determine the retention behavior of various plant cell wall derived oligosaccharides on the BEH Amide column a broad range of oligosaccharides was analyzed. The effect of variations in mobile phase composition and column temperature on the separation of different oligosaccharides was studied.

2.3.1 Influence of pH on separation of various oligosaccharides

The chromatograms in Figure 2.1A-C illustrate that both neutral and acidic oligosaccharides can be separated sufficiently when the mobile phase at pH 3 is used. The α-(1,4)-gluco-oligosaccharides are retained more strongly than the α-(1,5)-arabino-oligosaccharides. This confirms one of the selectivities that have been observed earlier in HILIC i.e. retention based on hydrophilicity, which results in correlation of retention times to the number of hydroxyl groups of the analytes *[29]*. Despite the fact that α-(1,5)-arabino-oligosaccharides with a DP up to 15 elute within 5 minutes, the separation is still reasonable. The acidic α-(1,4)-galacturono-oligosaccharides have a stronger retention due to their higher polarity.

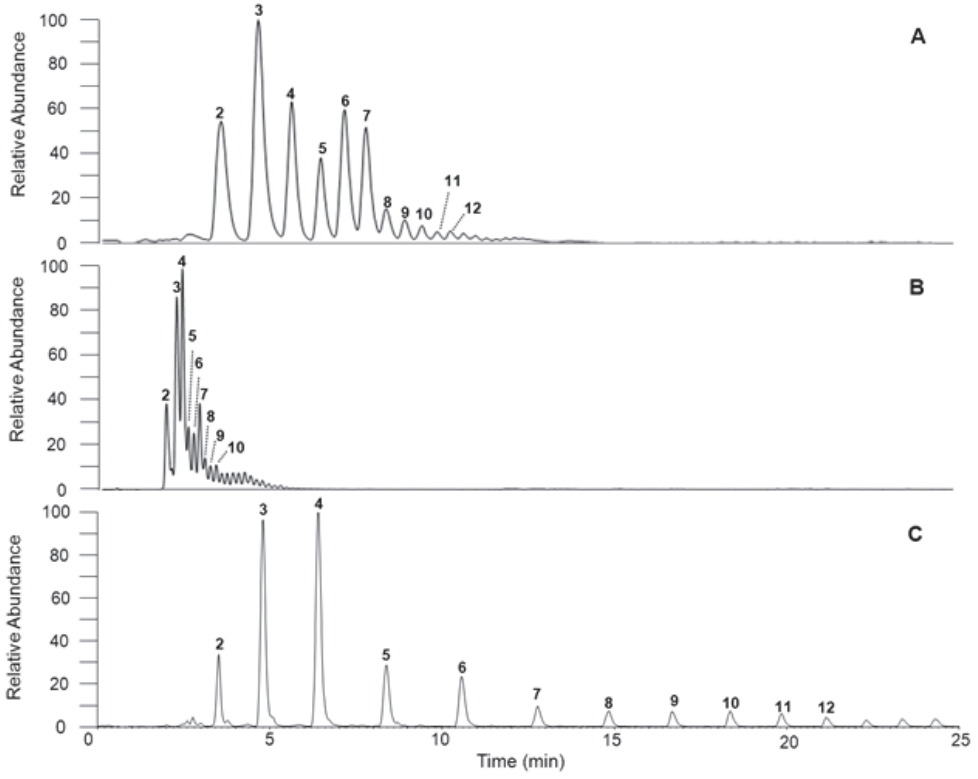


Figure 2.1. Elution pattern of various oligosaccharides on a BEH Amide column at pH 3 and 35 °C using MS-detection: (A) α-(1,4)-gluco-, (B) α-(1,5)-arabino-, (C) α-(1,4)-galacturono-oligosaccharides. Numbers indicate the degree of polymerization.

Figure 2.2 shows the effect of DP and type of oligosaccharide on the retention time at mobile phase conditions at pH 3. The α-(1,5)-arabino- and β-(1,4)-xylo-oligosaccharides, consisting of pentose sugars, are eluted significantly earlier compared to the α-(1,4)-gluco-, β-(1,4)-gluco-, β-(1,4)-manno- and β-(1,4)-galacto-oligosaccharides, which are composed of hexose sugars. When the mobile phase at pH 10 is used the retention behavior of neutral oligosaccharides is comparable to the elution pattern at pH 3 (results not shown). However, the acidic α-(1,4)-galacturono-oligosaccharides are hardly retained under these pH-conditions, which is also illustrated in Figure 2.2. This may be due to the fact that both surface silanol groups on the stationary phase and acidic analytes become negatively charged at high pH, resulting in ionic repulsion and less interaction with the stationary phase. When analyzing very complex mixtures it can be useful to run both at pH 3 and 10 in order to distinguish acidic from neutral oligosaccharides.
A mobile phase at pH 6.8 was also tested. Quite similar retention results compared to pH 3 were obtained (no further results shown). Since the combination of separation of acidic oligosaccharides and MS-response was best using the pH 3 mobile phase, further method development was carried out using this type of mobile phase.

2.3.2 Impact of column temperature on resolution

Adjusting column temperature is a frequently used step in method optimization. To evaluate the effect of varying column temperature on the separation of complex mixtures of oligosaccharides, several samples were analyzed using different column temperatures. In general, it was observed that retention of analytes decreased with increasing column temperature (data not shown). At a column temperature of 20 °C most of the components are adequately separated, but resolution decreases with increasing column temperature (data not shown). For complex mixtures it is, therefore, recommended to apply a low column temperature. A slight disadvantage of applying a low column temperature in combination with a mobile phase at pH 3 is the observation of undesired double peaks for neutral oligosaccharides due to α-/β-anomer separation. Anomeric peak-forming for neutral oligosaccharides can be reduced by increasing the column temperature or, alternatively, switching to a mobile phase at pH 10 (data not shown). No undesired double peaks were observed when applying a column temperature of 35 °C in combination with the mobile phase at pH 3 and the 40 minute gradient as described in section 2.2.2.

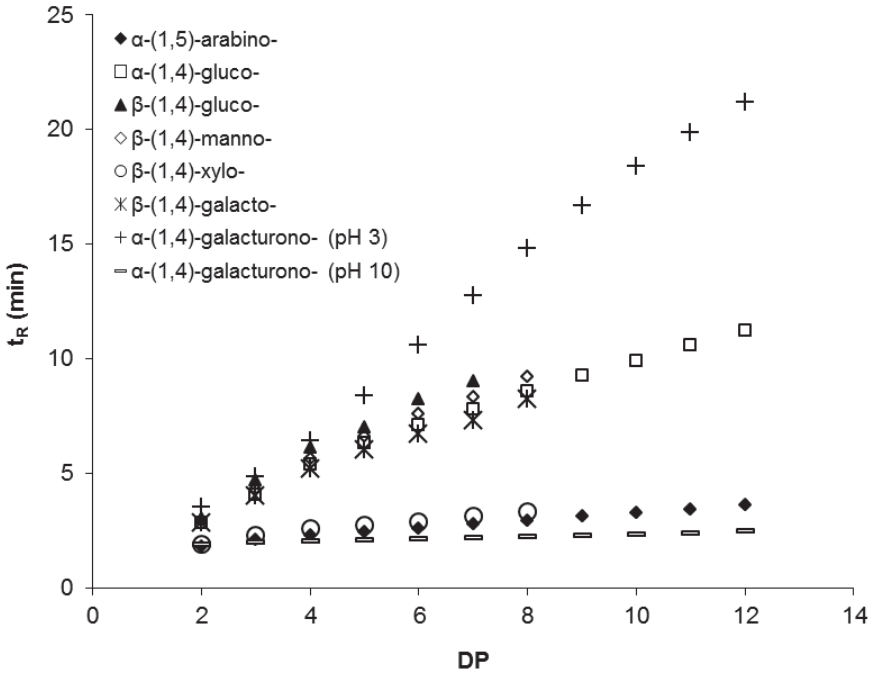


Figure 2.2. Retention time (t_R) plotted against the degree of polymerization (DP) for different oligosaccharides at a column temperature of 35 °C and mobile phase at pH 3. For α-(1,4)-galacturonic acid oligosaccharides the retention time is also plotted against the DP at mobile phase conditions at pH 10 to illustrate the typical difference in elution pattern compared to pH 3.

2.3.3 Separation of methyl esterified and acetylated galacturonic acid oligosaccharides

To evaluate if methyl esterified and acetylated galacturonic acid oligosaccharides can be separated by HILIC, samples of DM30- and sugar beet pectin digested with endo-polygalacturonase were analyzed. Figure 2.3 shows that partially methyl esterified galacturonic acid oligosaccharides can be separated effectively.

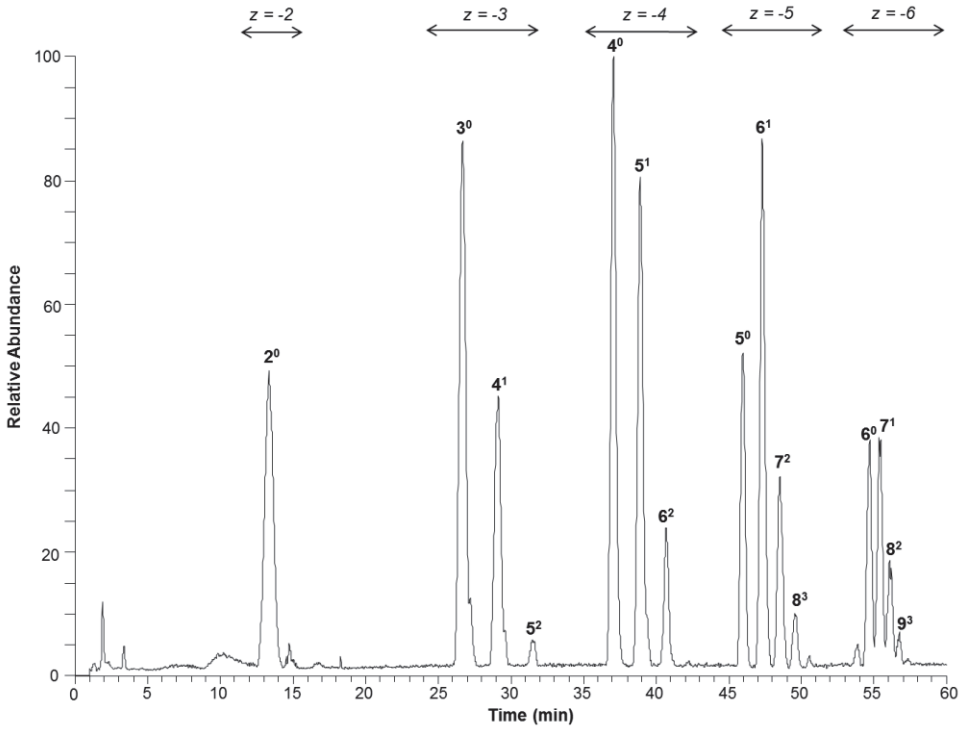


Figure 2.3. Base peak chromatogram of partially methyl esterified α-(1,4)-linked galacturonic acid oligosaccharides derived from DM30 pectin after treatment with endo-polygalacturonase on a Waters BEH Amide column (mobile phase at pH 3) using ESI-MS-detection in negative mode. Peaks are annotated with the DP and number of methyl esters (in superscript). Net charge (*z*) is also indicated. Adjusted gradient applied: linear from 80 to 56% (v/v) ACN/water in 60 min.

Separation was already obtained using the gradient described in section 2.2.2, but an optimized separation could be achieved by decreasing the steepness of the ACN/water-gradient (linear gradient over 60 min instead of 30 min), providing baseline separated peaks as illustrated in Figure 2.3. Easy annotation of the different peaks in the HILIC-chromatograms is possible using the *m/z*-values obtained by the MS. The retention time decreases significantly by the presence of methyl esters, most probably as a result of shielding a carboxyl group. Elution pattern is blockwise and is dominated by the net charge of the oligosaccharides. The separation of oligosaccharides carrying the same charge makes this method much more suitable for acidic oligosaccharide analysis than other existing methods

where elution is mostly dependent on the net charge of the analytes. *[4, 8, 12]*. Successful separation of methyl esterified oligogalacturonates with identical net charge using HPAEC-PAD at pH 5 was reported earlier *[30]*. The elution pattern was different from the one observed in HILIC. Baseline separation was not obtained and the large, more esterified oligomers eluted before the small less esterified oligomers when using HPAEC. This makes the HPAEC-chromatogram quite complex and less predictable compared to the HILIC elution behavior. Complete separation of partially methyl esterified galacturonic acid isomers, i.e. oligomers solely differing in methyl ester distribution was not obtained in our HILIC experiments. Information on the location of the methyl esters can be obtained by studying the fragmentation during MS^n-experiments, as will be demonstrated in section 2.3.5.

By enzymatic digestion of pectin with both endo-polygalacturonase and pectin lyase, a combination of unsaturated and saturated galacturonic acid oligosaccharides is obtained. Figure 2.4 shows the mass base peak chromatogram of such a mixture. On-line MS-coupling made it possible to identify the most abundant peaks, even when they were not completely separated. It is demonstrated that unsaturated structures are separated from saturated structures. The elimination mechanism through which the pectin lyase acts results in the loss of a hydroxyl group at the C4-position and the formation of an unsaturated galacturonic acid residue at the non-reducing end *[31]*. As described above, retention times correlate with the number of hydroxyl groups of the oligosaccharides. This explains that the unsaturated moieties elute earlier than the saturated ones. Some peaks in Figure 2.4 are very broad (e.g. peak for u4^3). It is expected that this is due to partial separation of isomers with a different methyl ester distribution. To be able to show isomers of interest extracted ion chromatograms can be an additional tool next to the use of base peak chromatograms.

Figure 2.5 shows the graphical correlation of DP, number of methyl esters attached and the effect of unsaturated structures on the retention time of galacturonic acid oligosaccharides on a BEH Amide column. The significant decrease in retention due to the presence of methyl esters and unsaturated galacturonic acid residues can be observed from this diagram, which is derived from experiments with mobile phase at pH 3 and the gradient as described in section 2.2.2.

To be able to evaluate the elution profile of acetylated oligosaccharides, a sugar beet pectin endo-polygalacturonase digest has been analyzed. ELS detection was used next to MS-detection to be able to evaluate if ELSD could be used routinely without MS for separation optimization and quantification. Next to this, ELSD might be more appropriate than MS for quantitative analysis of oligosaccharides since its response is based on concentration of analytes. The chromatograms are presented

in Figures 2.6A (ELSD) and 2.6B (MS). The peak pattern and relative peak intensities obtained by ELS- and MS-detection are rather comparable. Peak identification was possible using the MS-data. The presence of an acetyl group results in a significant decrease in retention, due to blocking of a hydroxyl group. Structure elucidation of acetylated oligogalacturonates has been described before but, in contrast to our method, required a combination of preparative anion-exchange and size-exclusion chromatography to purify the different oligogalacturonates before their structures could be determined by MS *[2]*.

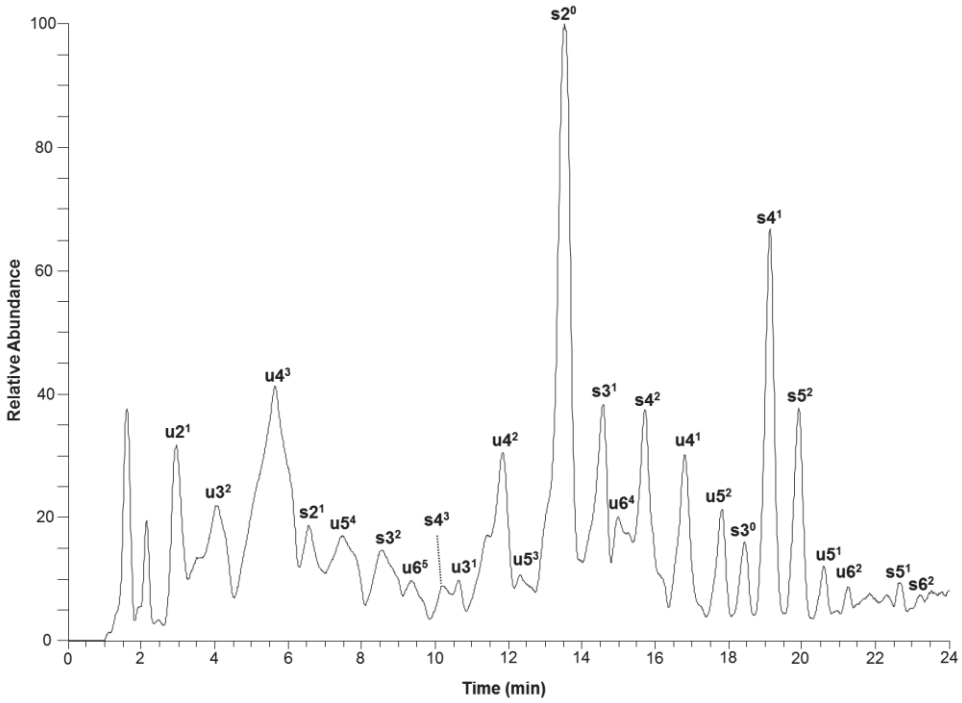


Figure 2.4. Base peak chromatogram of partially saturated, unsaturated and methyl esterified α-(1,4)-linked galacturonic acid oligosaccharides derived from DM56 pectin after treatment with endo-polygalacturonase and pectin lyase on a Waters BEH Amide column (mobile phase at pH 3) using ESI-MS-detection in negative mode. Peak annotation: u=unsaturated / s=saturated; DP; number of methyl esters (in superscript). Adjusted gradient applied: linear from 80 to 56% (v/v) ACN/water in 30 min.

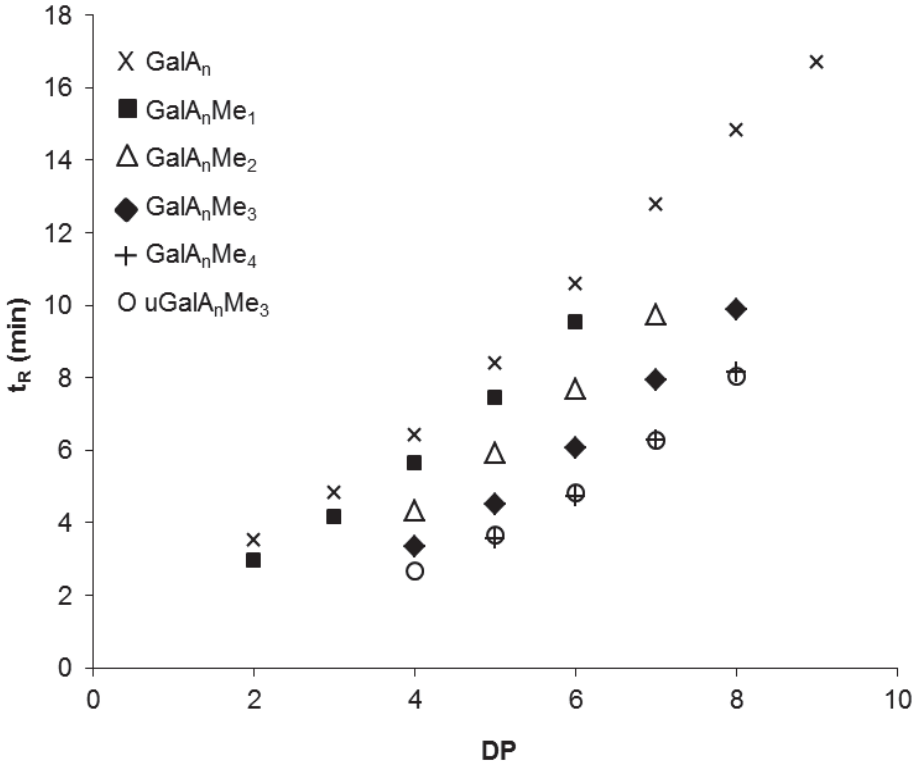


Figure 2.5. Effect of presence of methyl esters on the retention time (t_R) of unsaturated and saturated galacturonic acid oligosaccharides.

The improved understanding of the retention behavior of methyl esterified and acetylated galacturonic acid oligosaccharides obtained by the experiments carried out enables correct identification of peaks with the same *m/z*-values. For example, three methyl esters possess the same mass as one acetyl group. The effect on retention time, however, is completely different as explained above, which makes it possible to deduce the right structure. This feature is very useful in studying sugar beet pectin functionality since its derived oligosaccharides may contain both methyl and acetyl esters *[32]*.

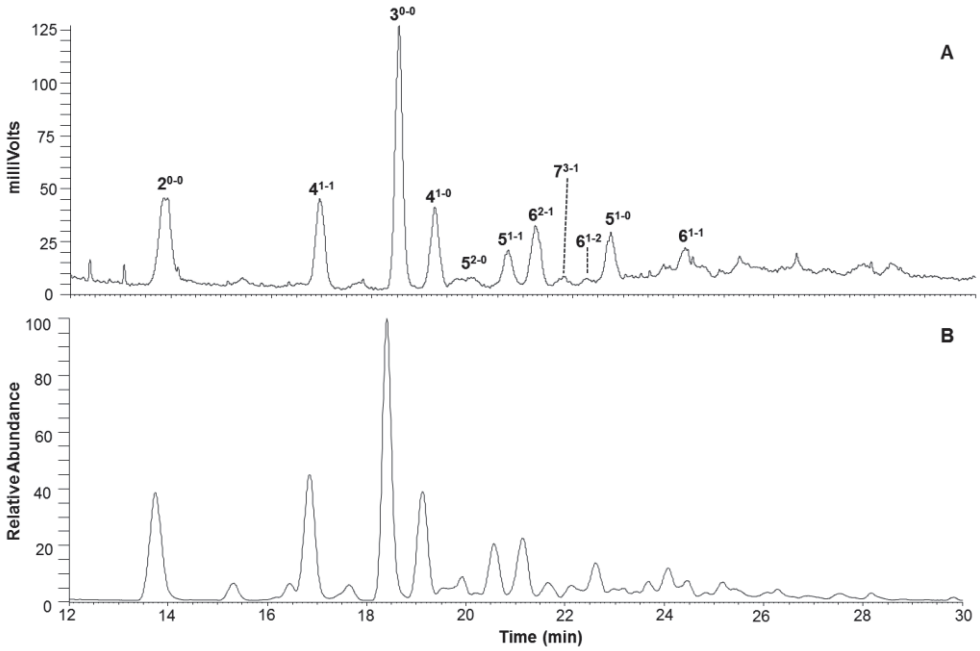


Figure 2.6. UPLC-elution profile of partially methyl esterified and acetylated α-(1,4)-linked galacturonic acid oligosaccharides derived from sugar beet pectin after treatment with endo-polygalacturonase on a Waters BEH Amide column (mobile phase at pH 3) using (A) ELSD and (B) ESI-MS-detection in negative mode (base peak chromatogram). Peak annotation: DP; number of methyl esters - number of acetyl groups (in superscript). Adjusted gradient applied: linear from 80 to 56% (v/v) ACN/water in 30 min.

2.3.4 Separation of neutral and acidic heteroglycan oligosaccharides

Oligosaccharides that are composed of different monosaccharides have also been analyzed. The BEH Amide column was capable of separating several heteroglycan oligosaccharide mixtures derived from xyloglucans, arabinoxylans, glucomannans, galactomannans, rhamnogalacturonans and xylogalacturonans. Adequate separations based on DP could be obtained in most cases. Isomeric structures, however, tend to co-elute, resulting in broad or overlapping peaks in the chromatogram (data not shown). The excellent separation potential of the BEH Amide column for acidic oligomers was again demonstrated during the analysis of xylogalacturonan and rhamnogalacturonan digests.

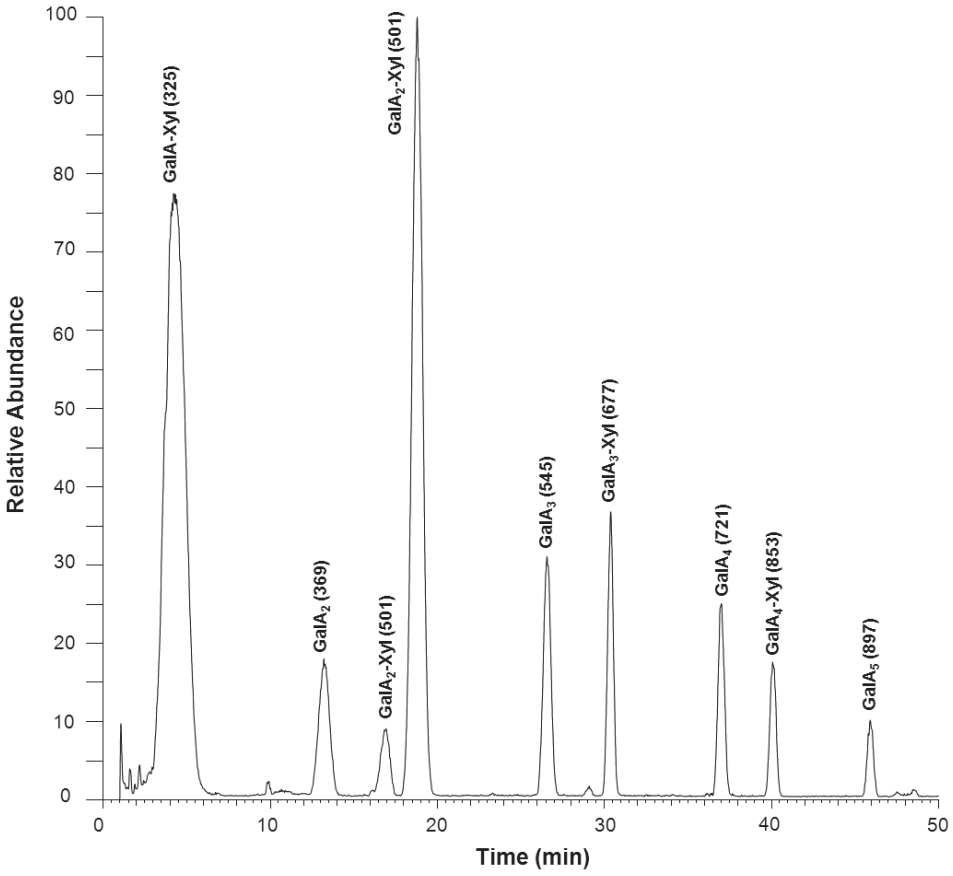


Figure 2.7. Base peak chromatogram of xylogalacturonan-oligosaccharides derived from *gum tragacanth* after treatment with xylogalacturonan hydrolase on a Waters BEH Amide column (mobile phase at pH 3) using ESI-MS-detection in negative mode. Peak annotation: GalA=galacturonic acid, Xyl=xylose, *m/z*-values in brackets. Adjusted gradient applied: linear from 80 to 56% (v/v) ACN/water in 60 min.

Figure 2.7 illustrates that acidic oligosaccharides carrying a neutral sugar side group (e.g. $GalA_3$-Xyl, *m/z* 677) can be separated from structures with the same charge (e.g. $GalA_3$, *m/z* 545). The higher number of hydroxyl groups involved in structures with a neutral substituent results in a stronger retention. Earlier research *[33]* has shown that oligosaccharides derived from enzymatic degradation of xylogalacturonan could be partially separated using HPAEC, but elution behaviors were difficult to predict, co-elution of several components was observed and

identification of unknown peaks required additional desalting and MALDI-TOF or electrospray MS-analysis.

2.3.5 Structure elucidation using HILIC-MS^n

The usefulness of MS as a tool for oligosaccharide structural characterization has been described before *[3, 12, 34-36]*. HILIC-MS^n has also proven to be a powerful tool in structure elucidation of unknown oligosaccharides in complex mixtures. In many cases the typical retention behavior of various oligosaccharides in combination with MS^n-data provides the required information for identification of unknown components. This is especially true for complex mixtures of saturated, unsaturated, methyl esterified and acetylated pectin derived structures as described in section 2.3.3. Figure 2.8A shows a chromatogram of a complex sugar beet hydrolysate. The steepness of the ACN/water gradient was adjusted (linear from 80 to 50% ACN/water (v/v) in 60 minutes) to be able to separate the large number of structures that were present in the sample. The chromatogram shows many peaks and MS^n-experiments were performed for identification of the most abundant peaks. MS^2- and MS^3-spectra are shown in Figures 2.8B and 2.8C, respectively, for the peak at 27.0 min (*m/z* 777). The MS-fragmentation pattern is indicated following the nomenclature according to Domon and Costello *[37]*. The assignment of Z- and C-type ions is based on similar assignments described in literature where these ions were found to be dominant over Y- and B-type ions when using negative ion mode *[38-41]*. From this information it was possible to annotate the unknown structure as an oligogalacturonate composed of four galacturonic acid monomers, one acetyl group and one methyl ester group. The location of the acetyl and methyl ester group was assessed from the observed cleavages at each MS step. The proposed structure derived from this is: GalA-AcGalA-GalA(OMe)-GalA. By studying the MS^n-data from the other peaks it was possible to identify the selected peaks in the chromatogram, as shown in Table 2.1. Valuable information on the composition of the hydrolysate could be obtained from this experiment. Some of the rhamnogalacturonan structures are unusual compared to findings reported earlier *[42, 43]*, especially for peak numbers 5 (methyl ester group within rhamnogalacturonan region) and peak numbers 7 and 8 (arabinose substituent near to galactose substituent), which is interesting for further investigation.

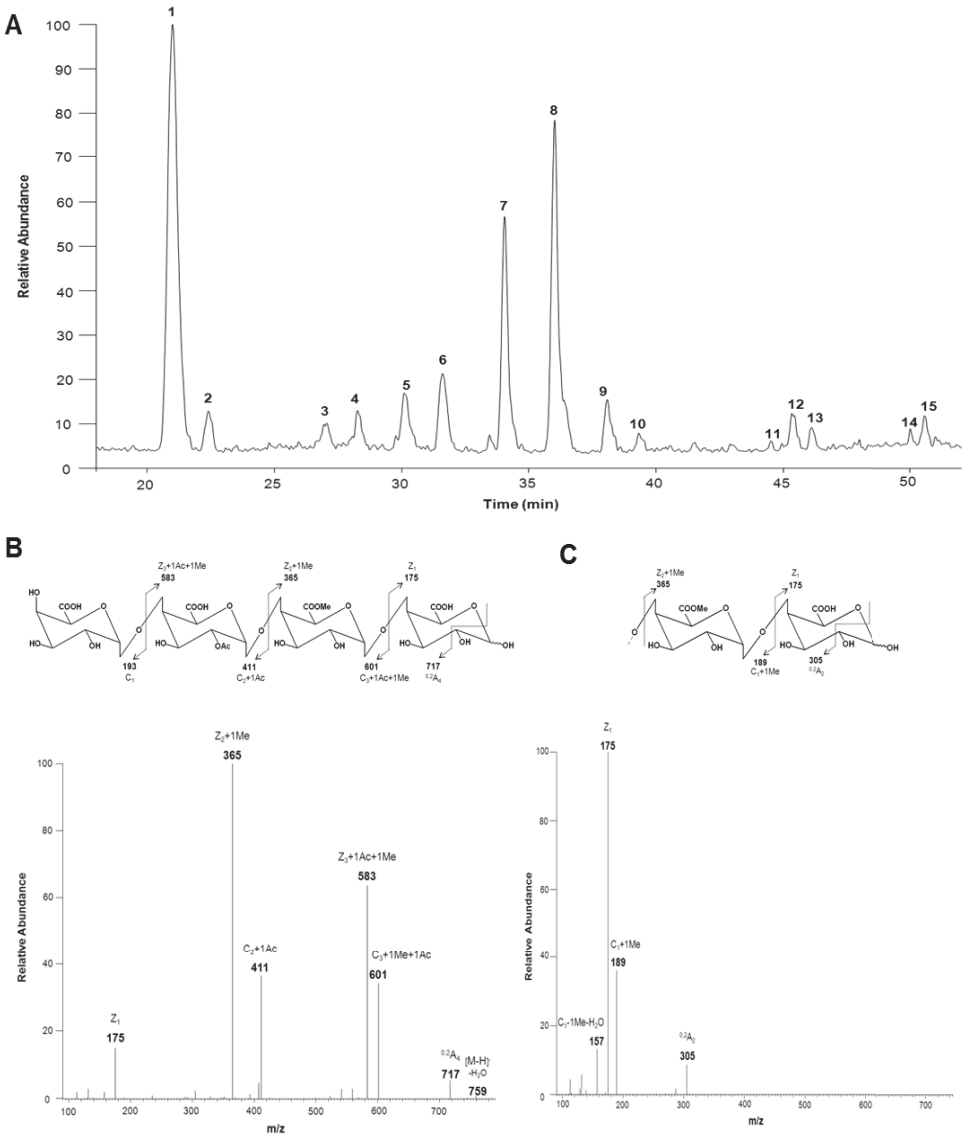
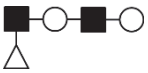
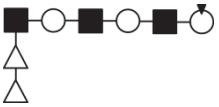
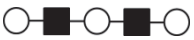
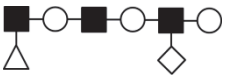
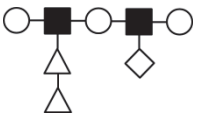
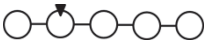
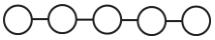
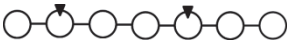
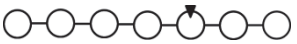


Figure 2.8. (A) Base peak chromatogram of sugar beet pulp hydrolysate Adjusted gradient applied: linear from 80 to 50% (v/v) ACN/water in 60 min.; (B) MS^2-experiment of peak 3 (*m/z* 777 → products) and chemical structure of *m/z* 777 and observed cleavages; (C) MS^3-experiment (*m/z* 777 → 365 → products) and chemical structure of major ion (*m/z* 365, derived from fragmentation of *m/z* 777) and observed cleavages.

Table 2.1. Annotation of peaks in Figure 2.8A.

peak number	t_R (min)	*m/z*	proposed structure
1	21.0	515	
2	22.4	661	
3	27.0	777	
4	28.2	823	
5	30.1	1321	
6	31.6	837	
7	34.0	1277	
8	36.0	1293	
9	38.1	721	
10	39.3	911	
11	44.5	897	
12	45.3	1087	
13	46.1	1277	
14	49.9	1073	
15	50.6	1263	

○, galacturonic acid; ■, rhamnose; △, galactose; ◇, arabinose; ▼, *O*-methyl; ▲, *O*-acetyl.

2.4 Conclusions

HILIC-ELSD-MS^n can be used for separation and characterization of a wide range of neutral and acidic plant cell wall derived oligosaccharides. It is demonstrated that acidic oligosaccharides with different DP but carrying the same charge can be separated adequately, giving this method unique benefits compared to other existing techniques like CE, RP and PGC liquid chromatography. Information is obtained on the typical retention behavior of different structural elements that can be found in plant cell wall analysis. Combining this information with MS^n-data enables efficient sequence identification of oligosaccharides in complex mixtures.

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Chapter 3

Enzymatic saccharification of sugar beet pulp for the production of galacturonic acid and arabinose; a study on the impact of the formation of recalcitrant oligosaccharides

Based on:

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Abstract

Enzymatic saccharification of sugar beet pulp was optimized on kg-scale to release the maximum amounts of monomeric galacturonic acid and arabinose with limited concomitant degradation of cellulose, using conditions that are feasible for industrial upscaling. A selected mixture of pectinases released 79% of the galacturonic acid and 82% of the arabinose as monomers from sugar beet pulp while simultaneously degrading only 17% of the cellulose. The recalcitrant structures that were obtained after hydrolysis were characterized using mass spectrometry. The most abundant structures had an average degree of polymerization of 4-5. They were identified as partially acetylated rhamnogalacturonan-oligosaccharides, mostly containing a terminal galacturonosyl residue on both reducing and non-reducing end, partially methyl esterified/acetylated homogalacturonan-oligosaccharides, mostly containing methyl and acetyl esters at contiguous galacturonosyl residues and arabinan-oligosaccharides, hypothesized to be mainly branched. It could be concluded that especially rhamnogalacturonan-galacturonohydrolase, arabinofuranosidase and pectin acetylesterase are lacking for further degradation of recalcitrant oligosaccharides.

3.1 Introduction

The production of monosaccharides by hydrolysis of polymeric carbohydrates in sugar beet pulp is a promising step towards increasing the value of this by-product of the sugar industry. The main constituent monosaccharides in sugar beet pulp are glucose (from cellulose), galacturonic acid and arabinose (both present in pectin). They constitute 60-70% of the dry matter of sugar beet pulp *[1]*. A large number of earlier studies mainly focused on production of monosaccharides from sugar beet pulp for bioethanol or biogas production *[e.g. 2, 3, 4]*. However, instead of serving as carbon source for fermentation, galacturonic acid and arabinose are interesting molecules for further conversion into building blocks which can be subsequently transformed into high-value biobased chemicals or materials, like polyesters, polyamides or plasticizers *[5]*. Moreover, the remaining cellulose after saccharification may be an interesting component for application as biobased fiber or nanocomposite material *[6]*. Therefore, it is desirable that cellulose degradation during hydrolysis is minimized, although some degradation will be beneficial to improve the physical accessibility of pectin to the enzymes *[7]*. Optimal release of galacturonic acid and arabinose from sugar beet pulp, while leaving cellulose intact, is a promising biorefinery opportunity. However, not much effort has been directed towards this so far.

The molecular structure of sugar beet pectin has been described extensively *[e.g. 8]*. Sugar beet pectin consists of homogalacturonan (HG), rhamnogalacturonan I (RG I) and, to a much lesser extent, rhamnogalacturonan II (RG II) regions. The degree of acetylation is about 35%, which is higher compared to pectins from other sources, with acetyl groups present in both HG and RG I. Linear β-(1,4)-linked galactan and highly branched arabinan, composed of α-(1,5)-linked backbones with α-(1,2) and/or α-(1,3) arabinofuranosyl substitutions, are side chains of RG I. The pectic arabinan content represents 20-25% of the sugar beet pulp dry matter. The galactan and arabinan side chains of RG I can be feruloylated *[9, 10]*.

Sugar beet pulp can be saccharified chemically or enzymatically. Since chemical saccharification results in high amounts of acid waste, substantial energy consumption, unspecific degradation of polysaccharides and partial breakdown of the released monosaccharides *[11, 12]*, enzymatic saccharification is considered as the most favorable approach. Previous work has already shown that a large set of enzymes is needed for complete degradation of sugar beet pectin to monosaccharides as enzyme resistant oligosaccharides are formed limiting the monosaccharide yield *[13]*. Although detailed structural information was obtained for feruloylated oligosaccharides, other enzyme resistant oligosaccharides were merely analyzed for their monosaccharide composition.

In this study, the enzymatic degradation of sugar beet pulp by a mixture of pectinases was investigated. Hydrolysis conditions were used that are feasible for industrial upscaling. Levels of galacturonic acid and arabinose release were measured and oligosaccharides resistant to enzymatic degradation were characterized in order to identify enzymes needed for further release of galacturonic acid and arabinose.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Chemicals

All chemicals used in this study were at least of analytical grade. Acetonitrile (ACN) was purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid, ammonium formate, heptafluorobutyric acid and methanesulfonic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Sulfuric acid, sodium hydroxide, ammonium hydroxide, sodium acetate, ammonium acetate, sodium carbonate and sodium hydrogen carbonate were purchased from Merck (Darmstadt, Germany). 2,5-Dihydroxybenzoic acid (DHB) was purchased from Bruker Daltonics (Bremen, Germany). Water was purified using a MilliQ-system (Millipore, Billerica, MA, USA).

3.2.1.2 Sugar beet pulp

Sugar beet pulp was obtained as pressed pulp from Suiker Unie (Dinteloord, The Netherlands), campaign 2009/2010 and had a dry matter content of 28.3% (w/w). The pulp was stored at −20 °C until the start of the experiments.

3.2.1.3 Enzymes

A series of enzyme preparations from various suppliers were tested previously for their ability to release galacturonic acid and arabinose from sugar beet pulp. Two enzyme preparations yielded the best results (not shown) and were therefore selected to study the hydrolysis experiments in more detail. Enzyme V, a commercial enzyme preparation from *Aspergillus aculeatus* (Viscozyme L), was obtained from Novozymes (Bagsvaerd, Denmark) and was used in combination with enzyme E, an experimental pectinase preparation from *Aspergillus niger,* obtained from Genencor (Rochester, NY, USA).

3.2.2 Enzyme digestions

A 10-liter container was filled with 2120 g of sugar beet pulp. After this, 5780 g of demineralized water and the enzyme solution (filled up to 100 mL with demineralized water) were added, resulting in a dry matter content of approximately 7.5% (w/w). The container was closed, mixed and placed in a water bath at 45 °C for saccharification. This temperature was indicated as optimum temperature by the enzyme suppliers. During incubation, the content of the container was mixed several times by shaking the container vigorously. After 6, 12, 24, 36 and 48 hours a homogeneous sample of approximately 1500 g of pulp suspension was removed from the container. This pulp suspension was pressed (300-400 bar) using a laboratory press (Hafico HP 2H, Fischer Maschinenfabrik, Neuss, Germany) and a 100-µm filter bag (Filter Specialists, Michigan City, IN, USA). The filter bag was wetted with demineralized water before use and rinsed with demineralized water thoroughly between each sample. The liquid fraction that was collected from the press was rapidly heated to the boiling point in a microwave to inactivate the enzymes and then directly frozen at –20 °C until further analysis. The pellet that was collected in the filter bag contained the insoluble material and was not heated in the microwave to avoid solubilization of polymeric structures. The pellet was placed directly in the freezer at −20 °C to stop further enzyme activity. The experiment was performed under three conditions, using (1) enzyme V only, (2) enzyme E only, or (3) both enzyme V and enzyme E. The enzyme concentration was 10.66 mL enzyme per kg dry matter. For the incubation with both enzymes (condition 3), 5.33 mL/kg dry matter of each enzyme was used.

3.2.3 Mass balance

The mass of sugar beet pulp that was added to the container before hydrolysis and the mass of all samples collected during and after the enzyme digestions were determined. The amount of water that was added to the system by wetting the filter bag was also measured by weighing the filter bag before and after pressing. Furthermore, it was determined that approximately 30 g of water (± 2%) evaporated while heating the hydrolysates in the microwave.

3.2.4 Dry matter content

The dry matter content of the samples was determined gravimetrically by drying for 18 h at 80 °C, followed by drying for 2 h at 105 °C until constant weight.

3.2.5 Protein content

The protein content (N × 6.25) of the samples was measured using an automated Kjeldahl distillation system (Kjeltec 2300, FOSS Analytical, Hilleroed, Denmark) after digestion of the samples in a FOSS Tecator digestion system.

3.2.6 Sugar composition

The monosaccharide composition of the solid and liquid fractions was analyzed before and after acid hydrolysis. Acid hydrolysis was performed using a slightly modified version of the procedure described previously *[14]*. In brief, 0.5 mL aqueous 72% (w/w) H_2SO_4 was added to 30-40 mg of dried sample in a reagent tube. The tube was placed in a heating block for 1 h at 30 °C, followed by the addition of 5 mL H_2O to obtain a concentration of 1 M H_2SO_4 and an additional 3 h heating step at 100 °C. The samples were then neutralized by adding 5 mL of 1 M NaOH and transferred to a 200-mL volumetric flask.
The arabinose, galacturonic acid, glucose, galactose, rhamnose, fructose, xylose, mannose, fucose and sucrose content of the samples was analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The HPAEC-PAD system (DX-600, Dionex, Sunnyvale, CA, USA; now Thermo Scientific, Waltham, MA, USA) was equipped with a CarboPac PA-1 column and a CarboPac PA-1 guard column (4.6 mm × 250 mm and 4.6 mm × 50 mm; Dionex). A flow rate of 1 mL/min was used and the column temperature was maintained at 30 °C. The injection volume was 25 µL. The mobile phase was composed of (A) H_2O, (B) 0.25 M NaOH and (C) 0.65 M NaOAc. The eluent profile was as follows: 0-15 min, isocratic 90% A and 10% B; 15-20 min, linearly to 70% A and 30% B; 20-30 min, linearly to 30% A, 30% B and 40% C; 30-32 min isocratic 30% A, 30% B and 40% C; 32-35 min, linearly to the initial conditions 90% A and 10% B; 35-45 min, isocratic 90% A and 10% B. The system was controlled using Empower software (Waters Corporation, Milford, MA, USA).

3.2.7 Organic acids

Acetic, lactic, citric, propionic, formic, malic and oxalic acid were measured according to the supplier's application note using an HPLC system with conductivity detection equipped with an Aminex HPX-87H analytical column (7.8 mm × 300 mm; Bio-Rad Laboratories, Hercules, CA, USA). An anion micromembrane suppressor (AMMS-ICE300, Dionex; 10 mM NH_4OH) was used to reduce the background conductivity. The column temperature was maintained at 45 °C and the flow rate was 0.6 mL/min. The injection volume was 50 µL. The mobile phase was composed of 1.6 mM heptafluorobutyric acid and elution was performed isocratically. An electrochemical detector (ED40, Dionex) was used in conductivity

mode and set at a range of 200 µS. The data were processed using Empower software (Waters Corporation).

3.2.8 Ash and salts

The ash content of the solid samples was determined gravimetrically by incinerating the samples for 3 h at 550 °C. The inorganic salt content in the liquid samples was measured using HPLC:
Cations (sodium, potassium, calcium, magnesium) were analyzed according to the supplier's application note using an HPLC system (DX-500, Dionex) with conductivity detection (ED40, Dionex). Elution was performed isocratically with 0.015 M methanesulfonic acid using an IonPac CS12A cation-exchange column (4.0 mm × 200 mm; Dionex) in combination with an IonPac CG12A guard column (4.0 mm × 50 mm; Dionex), both of which were maintained at 30 °C. The flow rate and injection volume were 1 mL/min and 25 µL, respectively. The detector was used in conductivity mode with the CSRS-Ultra suppressor set at 57 mA.
Anions (chloride, phosphate, sulphate, nitrate) were analyzed according to the supplier's application note using an HPLC system (DX-120, Dionex) with conductivity detection (ED40, Dionex). Elution was performed isocratically using a mobile phase composed of 2 mM sodium carbonate and 0.75 mM sodium hydrogen carbonate. The anions were separated using an IonPac AS9-SC carbonate eluent anion-exchange column (4.0 mm × 250 mm; Dionex) in combination with an IonPac AG9-SC guard column (4.0 mm × 50 mm; Dionex), both of which were at room temperature (approximately 20 °C). The flow rate was 1.5 mL/min and the injection volume was 25 µL. The detector was used in conductivity mode (range 30 µS, SRS 100 mA). The data were processed using Empower software (Waters Corporation).

3.2.9 Soluble oligo- and polysaccharides

The amounts of soluble oligo- and polysaccharides present in the hydrolysates (liquid fractions) were estimated by subtracting the amounts of mono- and disaccharides, protein, organic acids and salts from the dry matter content, considering that the amount of other soluble dry matter constituents is negligible *[15]*.

3.2.10 Cellulose degradation

The percentage of cellulose that was degraded during hydrolysis was calculated by comparing the amounts of glucose present in the pellets with the amount of glucose initially present in the sugar beet pulp, assuming that glucose in the pellets is mainly derived from cellulose.

3.2.11 Fractionation of the hydrolysates

The oligosaccharides present in the hydrolysates were fractionated using a modified version of the procedure described previously *[16]*. An Äkta purifier (GE Healthcare Life Sciences, Uppsala, Sweden) was equipped with three serially connected HiLoad 26/60 Superdex 30 prep-grade columns (GE Healthcare). The column was maintained at 35 °C. The freeze-dried hydrolysates (200 mg dissolved in 1.5 mL H_2O) were loaded onto the columns and isocratic elution was performed using 25 mM NH_4OAc as the mobile phase at a flow rate of 1.7 mL/min. A refractive index detector (RI-72, Showa Denko, Tokyo, Japan) was used to monitor the eluate. The system was controlled using Unicorn software (GE Healthcare). Sixty 6-ml fractions were collected from the hydrolysate that was obtained after 48 h of incubation. Forty 13-ml fractions were collected from the hydrolysate that was obtained after 12 h of incubation. Appropriate fractions were pooled and subsequently stored at −20 °C until further analysis.

3.2.12 Tentative identification of soluble oligo- and polysaccharides

The structure of the soluble oligo- and polysaccharides was tentatively identified on the basis of masses (*m/z*) detected with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS), using a slightly modified version of the procedure described previously *[17]*. Samples (1 µL each) were mixed with 1 µL of matrix solution (10 mg/mL DHB dissolved in 1 mL 30:70% (v/v) ACN/H_2O) on a stainless steel metal plate. The samples were crystallized under a stream of dry air and analyzed on an Ultraflextreme workstation (Bruker Daltonics) that was equipped with a 337-nm laser and controlled using FlexControl software (Bruker Daltonics). Analysis was performed in positive mode with a laser power intensity of 35-45%. Ions were detected using reflector mode. A mixture of maltodextrins (AVEBE, Veendam, The Netherlands; mass range 500-3500 Da) was used to calibrate the instrument. The data were analyzed using FlexAnalysis software (Bruker Daltonics). Peaks were tentatively annotated by calculating the masses (*m/z*) of structures that could theoretically be derived from sugar beet polysaccharides and comparing these with the detected *m/z*-ratios.

3.2.13 Oligosaccharide sequence identification

Sequencing of oligosaccharides was carried out using hydrophilic interaction chromatography coupled with mass spectrometry detection (HILIC-MS^n) . Analysis was performed as described previously *[18]*, using an adapted gradient. The mobile phase was composed of (A) H_2O, (B) ACN and (C) 200 mM ammonium formate with 2% (v/v) formic acid. The eluent profile was as follows: 0-60 min, linear from 15% A, 80% B and 5% C to 45% A, 50% B and 5% C; 60-68 min, linear to 15% A,

80% B and 5% C; 68-75 min, isocratic 15% A, 80% B and 5% C. A Velos Pro ion trap MS was used (Thermo Scientific). MS detection was performed in negative mode with the ion source voltage set to −4.5 kV, the capillary voltage set to −5 V, a capillary temperature of 350 °C, a heated ESI source temperature of 225 °C, a sheath gas flow rate of 40 (arbitrary units), an auxiliary gas flow rate of 12 (arbitrary units) and a sweep gas flow rate of 2 (arbitrary units).

3.3 Results and discussion

3.3.1 Enzymatic hydrolysis of sugar beet pulp

To obtain insight into the saccharification process, the amounts and types of components that were solubilized during enzymatic digestion were monitored. To this end, samples from the sugar beet pulp suspension were taken after several times of incubation. Pulp suspensions were separated into soluble (hydrolysate) and insoluble (pellet) fractions. The masses of all material added to or removed from the system were recorded in order to calculate the mass balance for the saccharification, after analysis of all fractions that were sampled.

3.3.1.1 Composition of hydrolysates

The composition of the hydrolysates obtained after 6, 12, 24, 36 and 48 h of incubation with enzyme mix V+E are shown in Table 3.1. The data for the individual enzymes can be found in Supplementary Tables 3.A1 and 3.A2. The results show that the monosaccharide levels in the hydrolysates gradually increased during the enzyme treatment. Galacturonic acid and arabinose made up approximately 50% of the hydrolysate dry matter content after 48 h of incubation. The amounts of galacturonic acid and arabinose released after 12 h and 24 h of incubation were approximately 50% and 80% of those released after 48 h, respectively. This shows that saccharification during the first 24 h of incubation is rapid. Soluble protein content also increased during hydrolysis, indicating protease side activity of the enzymes. This side activity may accelerate the physical accessibility of the pectin to the enzymes. The increase in organic acids during hydrolysis is expected to be caused by microbiological activity, since sugar beet pulp contains a diverse flora of acid-producing bacteria *[19]*.

3.3.1.2 Mass balance

The mass balance calculated for the enzymatic digestion using enzyme mix V+E is presented in Table 3.2. The recovery of total mass, dry matter and protein was close to 100%. During hydrolysis, sucrose was partially converted to glucose and fructose, which explains that sucrose recovery was low and fructose recovery

exceeded 100%. The recovery of organic acids also exceeded 100%, which can be attributed to the activity of acid-producing microorganisms during incubation. The recoveries of individual sugars ranged between 68% (rhamnose) and 91% (arabinose). The mass balance illustrates that the pectin derived monosaccharides were solubilized to a large extent, whereas glucose mainly remained in the insoluble fraction.

3.3.1.3 Arabinose and galacturonic acid yields

Figure 3.1 shows the yields of arabinose (Figure 3.1A) and galacturonic acid (Figure 3.1B) during hydrolysis as percentage of the amounts present in the sugar beet pulp. The amounts of soluble oligo- and polysaccharides present in the hydrolysates are illustrated in Figure 3.1C. It is clearly shown that enzyme mix V+E released 79% monomeric galacturonic acid and 82% monomeric arabinose after 48 h of hydrolysis. The galacturonic acid and arabinose yields after 48 h when using enzyme V only were 58% and 29%, respectively. When enzyme E was solely used, the galacturonic acid and arabinose yields after 48 h were 45% and 81%, respectively. Therefore, enzyme E proved to be very effective for the release of arabinose, while enzyme V released more galacturonic acid. Enzymes V and E showed a synergistic effect for the release of galacturonic acid. For arabinose, the synergistic effect was only visible during the first 24 h of incubation. The arabinose yields after 36 and 48 h of incubation using enzyme mix V+E were comparable to the arabinose yields using enzyme E.

A number of sugar beet pulp saccharification studies have already been described. Enzymatic pectin hydrolysis up to 95% monomers has been demonstrated *[20, 21]*, but this involved a high degradation of cellulose (> 60%), long enzyme incubation times (120 h) and a lower sugar beet pulp dry matter content (2.5-4%) compared to the present study (7.5%). Pectin hydrolysis with a monosaccharide yield of 70-80% and almost no cellulose degradation was obtained with incubation times of 24-48 h and a similar enzyme concentration as in this study *[22]*. However, the former required the use of a mix of many different commercial enzyme preparations and a low sugar beet pulp dry matter content of only 1%. Another study that described a 3% sugar beet pulp dosage and 48 h of incubation with a single commercial enzyme preparation yielded 71% galacturonic acid and 67% arabinose *[13]*. Hence, the yields obtained in the present study are higher compared to the yields presented previously with similar enzyme and sugar beet pulp dosages and relatively short incubation times, especially for arabinose.

Figure 3.1 indicates that further hydrolysis can be expected after 48 h. Based on the slope of the curve, an additional 5-10% yield with 24 h additional incubation time can be expected. Nevertheless, it might not be economically feasible to expand the hydrolysis time significantly for a large scale process.

Table 3.1. Composition of hydrolysates (liquid fractions) obtained from digestion with enzyme mixture V+E.

	t=6 h		t=12 h		t=24 h		t=36 h		t=48 h	
	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.
Dry matter	1.85	-	2.81	-	3.75	-	3.88	-	4.16	-
Arabinose	0.24	13.0	0.56	19.9	0.97	25.9	1.09	28.1	1.19	28.6
Galacturonic acid	0.23	12.4	0.44	15.7	0.70	18.7	0.80	20.6	0.91	21.9
Glucose	0.16	8.6	0.26	9.3	0.38	10.1	0.42	10.8	0.47	11.3
Galactose	0.04	2.2	0.08	2.8	0.14	3.7	0.16	4.1	0.19	4.6
Rhamnose	0.01	0.5	0.02	0.7	0.03	0.8	0.04	1.0	0.05	1.2
Fructose	0.09	4.9	0.12	4.3	0.15	4.0	0.16	4.1	0.18	4.3
Sucrose	0.28	15.1	0.21	7.5	0.18	4.8	0.14	3.6	0.12	2.9
Xylose	<0.01	<0.5	0.01	0.4	0.01	0.3	0.01	0.3	0.02	0.5
Mannose	<0.01	<0.5	0.01	0.4	0.01	0.3	0.02	0.5	0.02	0.5
Fucose	<0.01	<0.5	<0.01	<0.4	<0.01	<0.3	<0.01	<0.3	<0.01	<0.3
Protein	0.05	2.7	0.09	3.2	0.10	2.7	0.12	3.1	0.13	3.1
Salts	0.05	2.7	0.05	1.8	0.05	1.3	0.06	1.5	0.06	1.4
Organic acids	0.04	2.2	0.05	1.8	0.08	2.1	0.09	2.3	0.11	2.6
Soluble oligo- and polysaccharides[a]	0.66	35.7	0.91	32.4	0.95	25.3	0.77	19.8	0.71	17.1

[a] Estimated by subtracting amount of monosaccharides, sucrose, protein, salts and organic acids from dry matter content.

Table 3.2. Mass balance enzymatic sugar beet pulp digestion with enzyme mixture V+E.

Component	t=0 h		t=6 h		t=12 h		t=24 h		t=36 h		t=48 h			
		total amount sampled	pellet	hydrolysate	pellet	hydrolysate	pellet	hydrolysate	pellet	hydrolysate	pellet	hydrolysate	sum	recovery (%)
Sugar beet pulp	2120	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Added water	5880	n.a.	n.a.	82	n.a.	102	n.a.	77	n.a.	99	n.a.	59	n.a.	n.a.
Total mass	8000	7539	239	1358	256	1350	185	1396	168	1434	153	1419	7958	105.6
Dry matter	576	543	76	25	73	38	63	52	54	56	53	59	549	101.1
Protein	43.9	41.4	6.8	0.7	7.7	1.2	7.7	1.4	6.8	1.7	6.6	1.8	42.4	102.4
Ash/salts	39.9	37.6	4.6	0.7	4.8	0.7	6.8	0.7	6.6	0.8	7.6	0.9	34.1	90.7
Organic acids	1.0	0.9	0.1	0.5	0.1	0.7	0.1	1.1	0.1	1.3	0.1	1.6	5.6	625.6[a]
Sucrose	34.8	32.8	0.4	3.8	0.4	2.8	0.2	2.5	0.2	2.0	0.1	1.7	14.1	43.1[b]
Glucose	119.2	112.4	18.2	2.2	21.0	3.5	20.3	5.3	17.3	6.0	17.2	6.7	117.6	104.7[b]
Fructose	9.3	8.8	0.2	1.2	0.2	1.6	0.2	2.1	0.2	2.3	0.2	2.6	10.8	116.1[b]
Arabinose	108.9	102.6	13.5	3.3	10.6	7.6	5.6	13.5	3.6	15.6	3.0	16.9	93.3	90.9
Galacturonic acid	85.8	80.9	10.0	3.1	7.2	5.9	4.0	9.8	2.7	11.5	2.2	12.9	69.3	85.6
Galactose	29.9	28.2	4.2	0.5	3.7	1.1	2.4	2.0	1.8	2.3	1.7	2.7	22.3	79.1
Rhamnose	9.8	9.2	1.3	0.3	1.0	0.3	0.7	0.4	0.5	0.6	0.5	0.7	6.3	68.0
Xylose	7.5	7.1	1.3	0.1	1.4	0.1	1.3	0.1	1.2	0.1	1.3	0.1	7.2	101.5
Mannose	6.3	6.0	1.1	0.1	1.2	0.1	1.3	0.1	1.1	0.3	1.2	0.3	6.9	115.5
Fucose	2.1	2.0	0.1	<0.1	0.1	<0.1	0.1	<0.1	0.2	<0.1	0.2	<0.1	0.6	28.5
Others (e.g. lignin, fat)	77.0	72.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

All values expressed in grams.

Monosaccharide contents as analyzed after acid hydrolysis.

n.a., not applicable; n.d., not determined.

[a] Organic acids were formed during hydrolysis due to microbiological activity.

[b] During hydrolysis sucrose was partially converted to glucose and fructose.

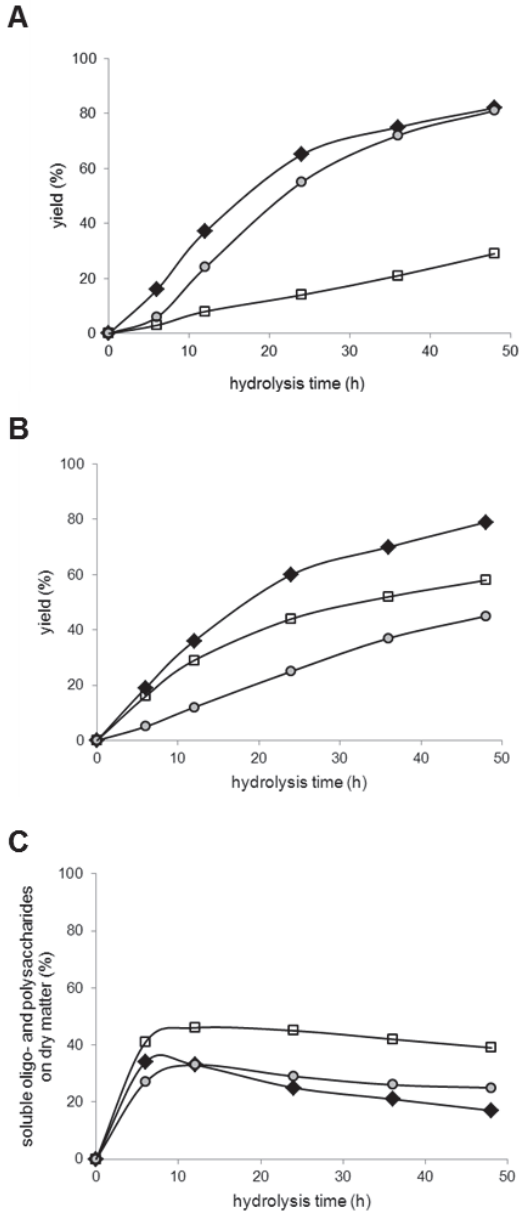

Figure 3.1. Release of (A) arabinose, (B) galacturonic acid and (C) soluble oligo- and polysaccharides during hydrolysis of sugar beet pulp. Legend: ◆, Enzyme V+E; ○, Enzyme E; ⊟, Enzyme V.

3.3.1.4 Cellulose degradation

As mentioned before, the formation of glucose from cellulose is undesired. The amount of cellulose that was degraded was estimated by analyzing the data in Table 3.2. This resulted in an estimation of cellulose degradation of 17% for enzyme mix V+E, 21% for enzyme V and 13% for enzyme E. Since some cellulose degradation is inevitable for obtaining high yields of pectin-derived monosaccharides, this might be an acceptable compromise. Nevertheless, further investigation is required to determine whether the mechanical properties of the remaining cellulose fibers are still adequate for application as bio-nanocomposite material *[23]*.

3.3.1.5 Oligo- and polysaccharide composition

The monosaccharide composition of the insoluble material (pellet) obtained during saccharification is shown in Figure 3.2A. Due to the activity of the pectinolytic enzymes, the amounts of pectin-derived monosaccharides in the insoluble pellets steadily decreased during hydrolysis. Since cellulose is only slightly hydrolyzed by the enzyme preparation, the pellets contain relatively high amounts of glucose at the end of the saccharification process. Next to high levels of monomeric galacturonic acid and arabinose, the hydrolysates also contained solubilized oligo- and polysaccharides. To analyze their constituent monosaccharides, the monosaccharide composition of the hydrolysates before and after acid hydrolysis was measured. Figure 3.2B illustrates that the soluble oligomeric and polymeric material consists mainly of arabinose, galacturonic acid and glucose, next to small levels of galactose and rhamnose. In order to provide information about the size and structure of these resistant oligo- and polysaccharides, they were fractionated and subsequently analyzed by MS. Such information is essential to reveal which enzymes are needed for further degradation of the recalcitrant structures. Furthermore, it provides insight into the enzyme activities that have contributed to the high galacturonic acid and arabinose yields.

3.3.2 Structural characterization of oligosaccharides

3.3.2.1 Fractionation and tentative identification of oligosaccharides

The hydrolysates were fractionated using preparative gel filtration chromatography, enabling separation of the oligosaccharides from monosaccharides, salts and proteins and partition of the oligosaccharides into various fractions for easier MS data collection and interpretation. Figure 3.3 shows the fractionation of the hydrolysates digested with enzyme mix V+E after 12 h and 48 h of incubation. The large peak at the end of the run represents the monosaccharides present in the hydrolysates, preceded by several peaks representing the various

oligosaccharides. The large initial peak represents polymeric structures, derived from proteins and polysaccharides.

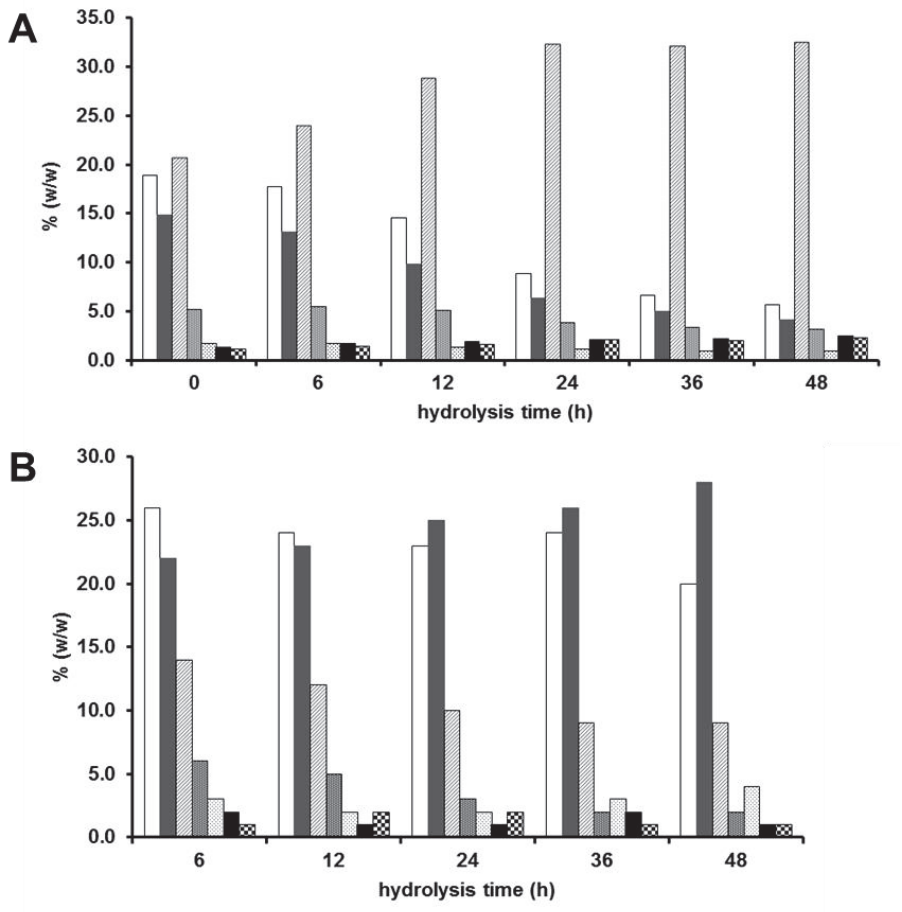

Figure 3.2. Monosaccharide composition of (A) insoluble pellets and (B) soluble oligo- and polysaccharides during enzymatic saccharification of sugar beet pulp with enzyme mixture V+E. Legend: □, arabinose; ■, galacturonic acid; ▨, glucose; ▩, galactose; ⁙, rhamnose; ■, xylose; ⧈, mannose.

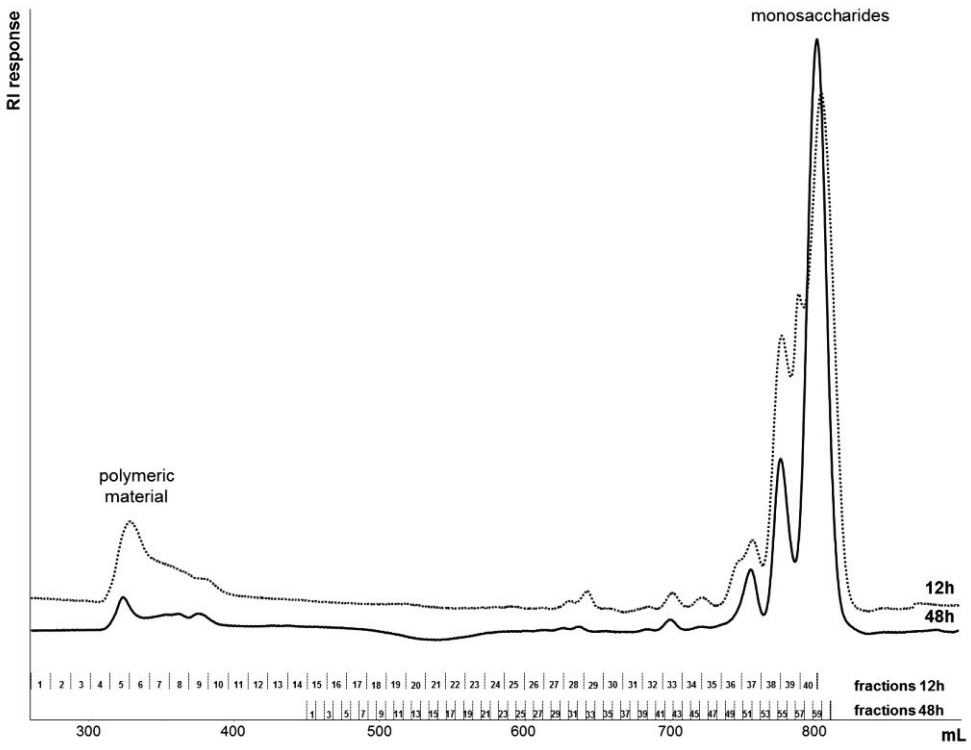

Figure 3.3. Preparative gel filtration chromatography (Superdex 30) fractionation of sugar beet pulp hydrolysates (liquid fraction) after 12 h and 48 h of enzymatic digestion with enzyme mixture V+E. Collected fraction numbers are illustrated.

The fractions that were collected by preparative gel filtration chromatography were subsequently analyzed by MALDI-TOF MS. Examples of mass spectra of Superdex fractions 33 and 28 (obtained after 12 h of incubation with enzyme mix V+E) and fractions 41 and 31 (obtained after 48 h of incubation with enzyme mix V+E) are presented in Figures 3.4 and 3.5, respectively. These time points were selected, since after 12 h of incubation approximately 50% of the hydrolysis had taken place (Figure 3.1), whereas samples after 48 h provide information on the structures obtained at the end of the incubation, thereby providing insight into the progress of the enzymatic degradation process. Fractions 33/41 and 28/31 are obtained after similar elution volumes (approximately 700 and 630 mL, respectively).

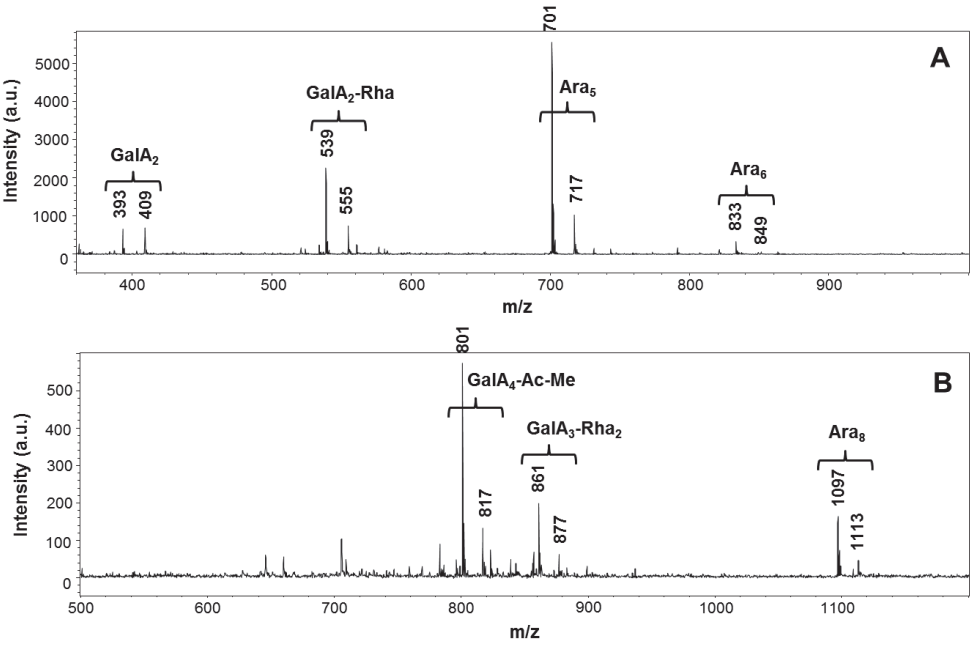

Figure 3.4. MALDI-TOF mass spectrum of fraction 33 (A) and fraction 28 (B); both obtained after 12 h of enzymatic digestion with enzyme mixture V+E. Peaks are tentatively annotated. Peak annotation: GalA, galacturonic acid; Ara, arabinose; Rha, rhamnose; Ac, acetyl group; Me, methyl ester. Both sodium and potassium adducts are visible for each component ($\Delta m/z$ = 16).

The MALDI-TOF mass spectra were relatively easy to interpret due to removal of impurities and salts during Superdex fractionation, which strongly reduced the number of peaks. Both sodium and potassium adducts were visible for each component ($\Delta m/z$ = 16). After 12 h of incubation with enzyme mix V+E, arabinan-, rhamnogalacturonan- and acetylated/methyl esterified homogalacturonan-derived oligosaccharides were present (Figure 3.4). Figure 3.5 shows that after 48 h of incubation with enzyme mix V+E, the arabinan-derived oligosaccharides were mostly degraded and the most abundant structures were partially acetylated rhamnogalacturonan- and acetylated/methyl esterified homogalacturonan-derived oligosaccharides.

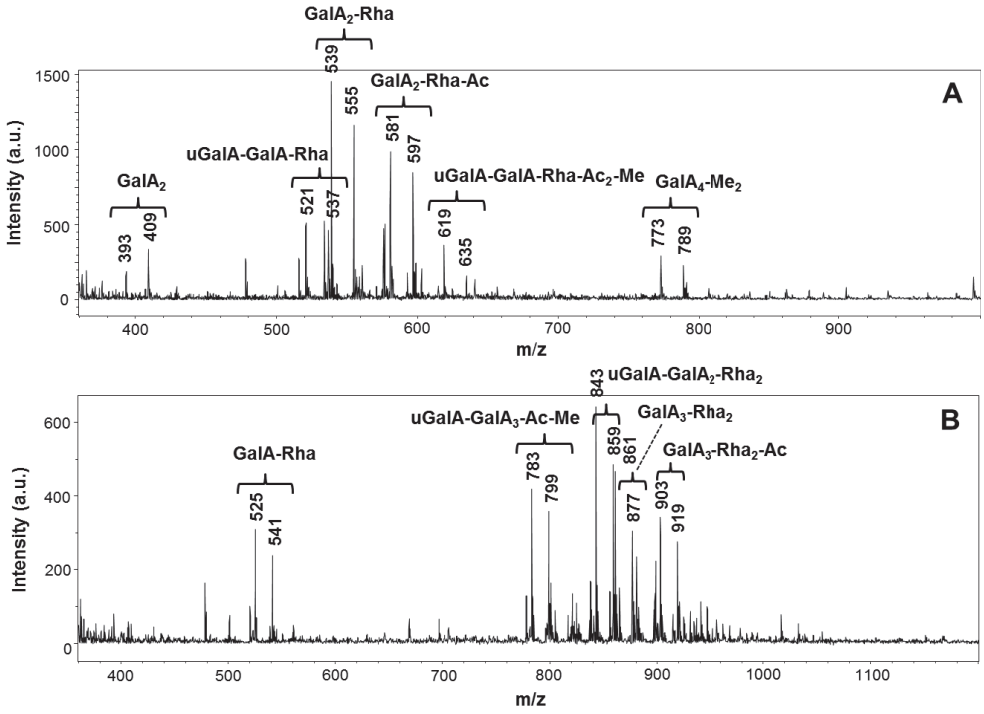

Figure 3.5. MALDI-TOF mass spectrum of fraction 41 (A) and fraction 31 (B); both obtained after 48 h of enzymatic digestion with enzyme mixture V+E. Peaks are tentatively annotated. Peak annotation: GalA, galacturonic acid; uGalA, unsaturated galacturonic acid; Ara, arabinose; Rha, rhamnose; Ac, acetyl group; Me, methyl ester. Both sodium and potassium adducts are visible for each component ($\Delta m/z = 16$).

In Tables 3.3 and 3.4, all oligosaccharides are summarized that were tentatively annotated using MALDI-TOF MS. Table 3.3 shows that after 12 h of incubation with enzyme V, arabinan-derived oligosaccharides with DP 3-10 were most dominantly present. The fractions obtained after 12 h of incubation with enzyme E were dominated by the presence of acetylated/methyl esterified homogalacturonan- and partially acetylated rhamnogalacturonan-derived oligosaccharides. Table 3.4 shows similar results for both enzymes after 48 h of incubation, except for the low average DP of the arabinan-oligosaccharides and a decreased presence of multiple acetylated/methyl esterified homogalacturonan-derived oligosaccharides. Unsaturated galacturonic acid residues were also present in several fractions.

The oligosaccharide sequence and the precise location of the methyl esters and acetyl groups could not be identified using MALDI-TOF MS. Therefore, HILIC-MS^n was applied as an additional tool.

Table 3.3. Oligosaccharide compositions analyzed in pooled fractions obtained after 12 h enzymatic digestion of sugar beet pulp with enzymes V and/or E. Tentatively annotated on the basis of masses (*m/z*) detected with MALDI-TOF MS.

Enzyme(s)	Fraction(s)	Oligosaccharide compositions
V+E	28-30	$GalA_4AcMe$, $GalA_3Rha_2$, Ara_8
	31-33	Ara_5, $GalA_2Rha$, Ara_6, $GalA_2$
	34-35	$GalA_2$, Ara_4, Ara_5
	36-38	Gal_3, Ara_3
E	24-25	$uGalAGalA_4Ac_3Me$, $uGalAGalA_4Ac_4Me$, $uGalAGalA_5Ac_2Me_2$, $uGalAGalA_5Ac_3Me_3$
	26-27	$GalA_4Rha_3$, $GalA_4Ac_2Me$, $GalA_5AcMe_2$, $GalA_5Ac_2Me_2$, $uGalAGalA_3Ac$
	28-31	$GalA_4AcMe$, $GalA_3Rha_2$, Ara_7, Ara_8
	32-33	$GalA_2Ac$, $GalA_2Rha$, Ara_5, Ara_6
	34-35	$GalA_2$, Ara_4
	36-38	Gal_3, Ara_3
V	23-26	Ara_{10}, $GalA_3Rha_2Ac$
	27	Ara_9
	28-31	Ara_8, $GalA_3Rha_2$, $uGalAGalA_3Rha_2Me$
	32-33	$GalA_2Rha$, Ara_5, Ara_6
	34-35	$GalA_2$, Gal_4, Ara_4
	36-38	Gal_3, Ara_3

Annotations: GalA, galacturonic acid; uGalA, unsaturated galacturonic acid; Ara, arabinose; Rha, rhamnose; Gal, galactose; Ac, acetyl group; Me, methyl ester.

3.3.2.2 Oligosaccharide sequence identification

The HILIC-patterns of the hydrolysates obtained after 12 h and 48 h of incubation with enzyme mix V+E are presented in Figure 3.6. The most abundant peaks were identified using MS^2- and MS^3-data in combination with the typical retention behavior of various oligosaccharides on the BEH Amide column as revealed before *[18, 24]*. Although a well resolved pattern for the crude hydrolysates could not be obtained, the HILIC-MS chromatogram in Figure 3.6A illustrates that after 12 h of hydrolysis, the main type of structures present were arabinan-, partially acetylated rhamnogalacturonan- and partially acetylated/methyl esterified homogalacturonan-oligosaccharides. The DP was in the range of 3-7. Figure 3.6B shows that after 48 h of hydrolysis, the arabinan-oligosaccharides were for a large part degraded and partially acetylated rhamnogalacturonan-oligosaccharides were most dominantly

present. The methyl esters and acetyl groups were located in contiguous galacturonosyl residues. Double acetylated galacturonosyl residues and galacturonosyl residues that are both methyl esterified and acetylated were not detected. It has been reported before that these structures are quite rare in sugar beet pectin *[25]*. The presence of oligosaccharides containing an unsaturated galacturonic acid residue, as suggested before by the interpretation of the MALDI-TOF mass spectra, was confirmed by the HILIC-MS-data. The unsaturated galacturonic acid residues were located at the non-reducing end of the oligosaccharides, indicating lyase activity of the enzymes.

Table 3.4. Oligosaccharide compositions analyzed in pooled fractions obtained after 48 h enzymatic digestion of sugar beet pulp with enzymes V and/or E. Tentatively annotated on the basis of masses (*m/z*) detected with MALDI-TOF MS.

Enzyme(s)	Fraction(s)	Oligosaccharide compositions
V+E	28-29	$uGalAGalA_2Rha_2$, $GalA_3Rha_2Ac$, $GalA_5Me_2$
	30-32	$uGalAGalA_2Rha_2$, $GalA_3Rha_2$, $uGalAGalA_3AcMe$, $GalA_3Rha_2Ac$, GalARha
	33-38	$GalA_3Rha_2$, $GalA_4AcMe$, $uGalAGalA_3AcMe$, $uGalAGalARha_2Gal$
	39-43	$GalA_2Rha$, $GalA_2RhaAc$, Ara_5, $GalA_4Me_2$, $uGalAGalARha$, $uGalAGalARhaAc_2Me$, $GalA_2$
	44-49	$GalA_2Rha$, $GalA_3Ac$, Ara_4
	50-53	Gal_3, Ara_3, Ara_2Gal
E	25-29	$GalA_4AcMe$, $GalA_3Rha_2$, $uGalAGalA_2Rha_2$
	30-33	$GalA_2Ac$, $GalA_2Rha$, $GalA_2RhaAc$, $GalA_3AcMe$, $GalA_2Ac_2$
	34-35	$GalA_2$, $GalA_3Ac$, Ara_5
	36-38	Gal_3, Ara_3, $GalA_2Me$
V	26-27	Ara_{10}, $GalA_3Rha_2Ac$
	28	Ara_9
	29-31	Ara_8, $GalA_3Rha_2$, $uGalAGalA_3Rha_2Me$, Ara_7
	32-33	$GalA_2Rha$, Ara_5, Ara_6
	34-35	$GalA_2$, Gal_4, Ara_4
	36-38	Gal_3, Ara_3

Annotations: GalA, galacturonic acid; uGalA, unsaturated galacturonic acid; Ara, arabinose; Rha, rhamnose; Gal, galactose; Ac, acetyl group; Me, methyl ester.

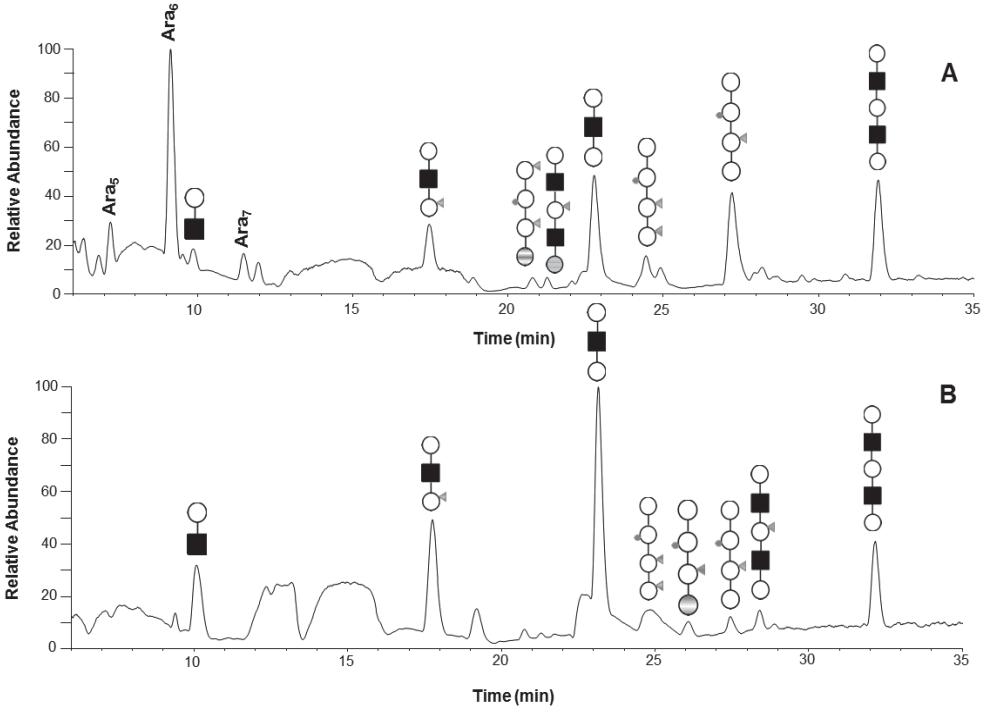

Figure 3.6. HILIC-MS chromatogram of sugar beet pulp hydrolysate (liquid fraction obtained after digestion with enzyme mixture V+E) after 12 h (A) and 48 h (B). Peak annotation: ○, galacturonic acid; ◍, unsaturated galacturonic acid; ■, rhamnose; ▲, acetyl group; ●, methyl ester; Ara, arabinose.

3.3.3 Enzyme activities

Based on the results, important information could be deduced about enzyme activities that contributed to the obtained galacturonic acid and arabinose yields and, subsequently, lacking enzyme activities for further hydrolysis of the recalcitrant oligosaccharides. From the HILIC-MS-data, it was concluded that the rhamnogalacturonan-oligosaccharides that were present in the hydrolysates mostly contained a galacturonic acid residue on both the reducing and non-reducing end. This indicates that besides activity of RG-hydrolase, RG-rhamnohydrolase must have been active, since only this enzyme removes terminal rhamnosyl residues from the non-reducing end of RG and RG-oligosaccharides *[26]*. RG-galacturonohydrolase might be helpful for further degradation of the RG-oligosaccharides, since it removes terminal galacturonosyl residues from the non-

reducing end *[27]*. Pectate lyase and RG-lyase activity were also observed from the presence of unsaturated galacturonic acid residues at the non-reducing end of HG- and RG-oligosaccharides. Since unsaturated galacturonic acid cannot be converted in its saturated form anymore after the enzymatic hydrolysis, the lyase activity has a negative effect on the galacturonic acid yield.
Endo- and exo-polygalacturonase activity could be deduced from the presence of HG-oligosaccharides with low DP and the release of monomeric galacturonic acid. Particularly for enzyme E, a lack of pectin acetylesterase activity was observed since several acetylated structures were identified in the saccharification end-products. It is known that the presence of acetyl groups hampers the enzyme action of polygalacturonase and pectin methylesterase on the substrate *[28, 29]*. The HG-oligosaccharides that were identified after 48 h of incubation with enzyme mix V+E mostly contained methyl esters and acetyl groups, indicating that pectin acetylesterase was hindered by the presence of methyl esters, an observation which has been reported previously *[30]*. Therefore, addition of a pectin acetylesterase, which is able to remove acetyl groups from HG in the presence of methyl esters would be most favorable for further hydrolysis of the recalcitrant HG-oligosaccharides.
The RG-oligosaccharides were devoid of arabinan or galactan side chains, indicating activity of (endo- and exo-)arabinanase(s) and (endo- and exo-) galactanase(s) during hydrolysis. The remaining arabino-oligosaccharides after enzymatic hydrolysis are hypothesized to be most dominantly branched as it is known that these are more difficult to degrade than linear arabinan-oligomers *[31]*. This indicates a lack of arabinofuranosidase activity. Additional in-depth characterization would be required to confirm the branching configuration, e.g. by using porous-graphitized carbon liquid chromatography with MS detection as decribed before *[32]* or NMR.

3.4 Conclusions

This study demonstrated that enzymatic hydrolysis of sugar beet pulp, using conditions that are feasible for industrial application, released 79% of the galacturonic acid and 82% of the arabinose that is present in sugar beet pulp while simultaneously degrading approximately 17% of the cellulose. Possibilities that have been identified for further increase of galacturonic acid and arabinose yields are extension of the hydrolysis time, addition of RG-galacturonohydrolase, arabinofuranosidase or pectin acetylesterase active in the presence of methyl esters. This saccharification process provides a promising biorefinery opportunity for sugar beet pulp, alternative to its use for biofuel production.

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Supplementary Table 3.A1. Composition of hydrolysates (liquid fractions) obtained from digestion with enzyme V.

	t=6 h		t=12 h		t=24 h		t=36 h		t=48 h	
	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.
Dry matter	1.54	-	2.23	-	2.89	-	3.20	-	3.46	-
Arabinose	0.05	3.2	0.12	5.4	0.21	7.3	0.32	10.0	0.44	12.7
Galacturonic acid	0.20	13.0	0.36	16.1	0.52	18.0	0.62	19.4	0.68	19.7
Glucose	0.10	6.5	0.14	6.3	0.18	6.2	0.20	6.3	0.23	6.6
Galactose	0.03	1.9	0.05	2.2	0.09	3.1	0.10	3.1	0.12	3.5
Rhamnose	0.01	0.6	0.01	0.4	0.02	0.7	0.03	0.9	0.03	0.9
Fructose	0.07	4.5	0.09	4.0	0.10	3.5	0.11	3.4	0.12	3.5
Sucrose	0.27	17.5	0.21	9.4	0.19	6.6	0.18	5.6	0.17	4.9
Xylose	<0.01	<0.5	<0.01	<0.4	<0.01	<0.3	<0.01	<0.3	<0.01	<0.3
Mannose	<0.01	<0.5	<0.01	<0.4	<0.01	<0.3	<0.01	<0.3	<0.01	<0.3
Fucose	<0.01	<0.5	<0.01	<0.4	<0.01	<0.3	<0.01	<0.3	<0.01	<0.3
Protein	0.08	5.2	0.10	4.5	0.11	3.8	0.13	4.1	0.14	4.0
Salts	0.07	4.5	0.08	3.6	0.09	3.1	0.09	2.8	0.09	2.6
Organic acids	0.03	1.9	0.05	2.2	0.09	3.1	0.10	3.1	0.12	3.5
Soluble oligo- and polysaccharides[a]	0.63	40.9	1.02	45.7	1.29	44.6	1.32	41.3	1.32	38.2

[a] Estimated by subtracting amount of monosaccharides, sucrose, protein, salts and organic acids from dry matter content.

Supplementary Table 3.A2. Composition of hydrolysates (liquid fractions) obtained from digestion with enzyme E.

	t=6 h		t=12 h		t=24 h		t=36 h		t=48 h	
	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.	%(w/w) as is	%(w/w) on d.m.
Dry matter	1.03	-	1.87	-	2.92	-	3.59	-	4.00	-
Arabinose	0.10	9.7	0.38	20.3	0.82	28.1	1.07	29.8	1.21	30.3
Galacturonic acid	0.06	5.8	0.15	8.0	0.30	10.3	0.43	12.0	0.53	13.3
Glucose	0.11	10.7	0.20	10.7	0.31	10.6	0.41	11.4	0.47	11.8
Galactose	0.01	1.0	0.04	2.1	0.09	3.1	0.14	3.9	0.17	4.3
Rhamnose	<0.01	<1.0	0.01	0.5	0.02	0.7	0.03	0.8	0.04	1.0
Fructose	0.09	8.7	0.13	7.0	0.17	5.8	0.19	5.3	0.21	5.3
Sucrose	0.28	27.2	0.19	10.2	0.12	4.1	0.08	2.2	0.05	1.3
Xylose	<0.01	<1.0	<0.01	<0.5	0.01	0.3	0.01	0.3	0.01	0.3
Mannose	<0.01	<1.0	<0.01	<0.5	0.01	0.3	0.01	0.3	0.01	0.3
Fucose	<0.01	<1.0	<0.01	<0.5	<0.01	<0.3	<0.01	<0.3	<0.01	<0.3
Protein	0.04	3.9	0.06	3.2	0.09	3.1	0.11	3.1	0.15	3.8
Salts	0.05	4.9	0.06	3.2	0.08	2.7	0.09	2.5	0.10	2.5
Organic acids	0.02	1.9	0.04	2.1	0.06	2.1	0.07	1.9	0.10	2.5
Soluble oligo- and polysaccharides[a]	0.24	23.3	0.61	32.6	0.84	28.8	0.95	26.5	0.95	23.8

[a] Estimated by subtracting amount of monosaccharides, sucrose, protein, salts and organic acids from dry matter content.

Chapter 4

In vitro fermentability of sugar beet pulp derived oligosaccharides using human and pig fecal inocula

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Abstract

The *in vitro* fermentation characteristics of different classes of sugar beet pectic oligosaccharides (SBPOS) were studied using human and pig fecal inocula. The SBPOS consisted mainly of partially acetylated rhamnogalacturono-oligosaccharides and partially methyl esterified/acetylated homogalacturono-oligosaccharides. Some SBPOS contained an unsaturated galacturonic acid residue at their non-reducing end. It was shown that SBPOS could be completely fermented by human and pig fecal microbiota, thereby producing butyrate, yet mainly acetate and propionate as metabolites. The degradation of SBPOS by pig fecal microbiota was different and much slower compared to human fecal microbiota. In general, rhamnogalacturono-oligosaccharides were slower degraded than homogalacturono-oligosaccharides. Acetylation of rhamnogalacturono-oligosaccharides lowered the degradation rate by pig fecal microbiota, but not by human fecal microbiota. No classic bifidogenic effect was shown for SBPOS using human fecal inoculum. However, several other potentially interesting modifications in the microbiota composition that can be associated with host health were observed, which are discussed.

4.1 Introduction

In recent years, generation of prebiotics from plant biomass sources has gained much attention *[1-3]*. Sugar beet pectin derived oligosaccharides have been described as one of the promising prebiotic candidates. They can be produced from sugar beet pulp, which is a large volume by-product of the beet sugar industry. Several studies have indicated prebiotic functionality of these types of oligosaccharides, i.e. selective fermentation by gastrointestinal microbiota *[4-6]*. This stimulates the growth and/or activity of intestinal bacteria (e.g. bifidobacteria, lactobacilli) that are associated with host health and well-being. Besides this, formation of fermentation products like gases (mainly CO_2 and H_2), short chain fatty acids (SCFA; mainly acetate, propionate and butyrate) and lactate occurs *[5, 6]*. It is believed that especially butyrate production is beneficial to host health as it is the most important fuel for colonocytes, it effects the development and gene expression in intestinal cells and has a protective role against ulcerative colitis and colon cancer *[7-9]*.

Sugar beet pectin derived oligosaccharides are potential ingredients for functional foods or feed. Previous studies described tailored enzymatic production of specific classes of oligosaccharides from sugar beet pulp, e.g. arabino-oligosaccharides *[4]* and homogalacturono- and rhamnogalacturono-oligosaccharides *[5]*. However, large scale production of these oligosaccharides will be costly due to downstream processing and disposal of remaining material. Hence, it would be more favorable if sugar beet pectin derived oligosaccharides could be produced in combination with the extraction of other valuable components.

Recently, an enzymatic saccharification process has been described *[10]* in which mainly sugar beet pectin was targeted to release high amounts of its monosaccharides, like galacturonic acid and arabinose. However, apart from the monosaccharides released, sugar beet pectin derived oligosaccharides were obtained that had resisted enzymatic hydrolysis. They were characterized as partially acetylated rhamnogalacturono-oligosaccharides, mostly containing a terminal galacturonosyl residue on both reducing and non-reducing end, partially methyl esterified and/or acetylated homogalacturono-oligosaccharides and (branched) arabino-oligosaccharides. Earlier studies on *in vitro* fermentation of pectin *[11, 12]* and pectic oligosaccharides *[13]* showed that pectic oligosaccharides are more difficult to degrade by human and animal colonic bacteria compared to other nondigestible oligosaccharides. This might result in fermentation of a large part of these oligosaccharides in the more distal part of the colon, which might be helpful to decrease the risk of the formation of potentially toxic products (e.g. ammonia, polyamines) due to a switch from saccharolytic to proteolytic fermentation *[14]*. Application of these types of oligosaccharides as

ingredient for functional foods or feed, in addition to the production of sugar beet pectin derived monosaccharides, would greatly increase the biorefinery opportunities for sugar beet pulp. So far, little has been reported on the prebiotic potential and fermentation characteristics of the complex mixture of oligosaccharides, which is obtained after enzymatic saccharification of sugar beet pulp.
Therefore, the aim of this work was to study the *in vitro* fermentation characteristics of the oligosaccharides obtained after enzymatic saccharification of sugar beet pulp by using fecal inocula from humans and pigs. Moreover, the fate of the different types of oligosaccharides and the changes in human fecal bacterial populations during *in vitro* fermentation were studied. Fructo-oligosaccharides (FOS) were selected as a reference substrate for the *in vitro* fermentation experiments, since these are one of the most frequently described prebiotic oligosaccharides and much is already known about their *in vitro* fermentation behavior.

4.2 Materials and methods

4.2.1 Chemicals

All chemicals used in this study were at least of analytical grade.

4.2.2 Substrates

Fructo-oligosaccharides (FOS, Frutalose OFP) were obtained from Sensus (Roosendaal, The Netherlands) and sugar beet pectic oligosaccharides (SBPOS) were obtained from Cosun Biobased Products (Breda, The Netherlands).

4.2.3 Culture medium

The culture medium was based on the modified standard ileal efflux medium (SIEM) as described before *[15]*. All medium components were obtained from Tritium Microbiology (Veldhoven, The Netherlands). The pH of the medium was adjusted to 6.0 using MES buffer.

4.2.4 Fecal inoculum

4.2.4.1 Human fecal inoculum

A standardized pool of adult fecal inoculum was prepared as described and validated before *[15, 16]*. The fecal samples used to produce the standardized inoculum were pooled and were obtained from 10 healthy European adults who did not receive antibiotics in the 2 months before donation. The ratio of males to

females in the human subject pool was 4:1. Their mean age was 44.7 years with a standard error of the mean of 3.54 years. Their mean body mass index (BMI) was 23.3 kg/m^2 with a standard error of the mean of 0.85 kg/m^2.

4.2.4.2 Pig fecal inoculum

Three multiparous non-lactating sows (Dutch Landrace, aged 4 years) were used in this study. Their mean body weight was 233.0 kg with a standard error of the mean of 10.02 kg. The sows did not receive any antibiotics for at least 2 months prior to feces collection. The sows were individually housed in pens at the experimental facilities of Wageningen University. The sows were fed twice daily a commercial pelleted maize- and wheat-based maintenance diet (AgruniekRijnvallei, Lienden, The Netherlands). Immediately after defecation, feces were collected in a plastic container prefilled with CO_2. Extra CO_2 was decanted into the container with feces to ensure anaerobic storage conditions. The container with feces was closed and immediately placed in an insulated box containing crushed ice. After pooling, the feces were diluted six times (w/v) with sterilized 0.9% (w/v) NaCl solution and subsequently homogenized and filtered using a filter bag (type BA6041/STR, Seward, Worthing, UK) and a stomacher (model Classic 400, Seward). The filtered fecal solution was subsequently added to the culture medium with a ratio of 5:82 (v/v) to obtain the final inoculum. All liquids were kept at 39 °C under anaerobic conditions. Inoculum was prepared within 60 minutes after feces collection. All experimental procedures were performed in compliance with the appropriate rules and institutional guidelines and were approved by Wageningen University.

4.2.5 *In vitro* fermentations

Substrates (100 mg) were weighed into 20-mL flasks. Next, 9 mL of prewarmed culture medium and 1 mL of inoculum was added to the flasks. Control samples without added substrate were used to monitor the background fermentation. All handling was done in an anaerobic cabinet (gas phase: 96% N_2 and 4% H_2) to maintain anaerobic conditions. Flasks were closed with rubber stoppers and aluminium caps and were put in an incubator shaker (Innova 4000, New Brunswick Scientific, Enfield, CT, USA) at 100 rpm and 37 °C (human inoculum) or 39 °C (pig inoculum). After different time points of incubation, the flasks were opened and the liquid was divided over two test tubes. One of these tubes was immediately frozen in liquid nitrogen and stored at −80 °C to preserve the sample for microbiological analysis (only for samples with human inoculum). The other tube was put into a boiling water bath for 5-10 minutes. This sample was used for analysis of pH, SCFA, lactate and oligosaccharide degradation. The experiments with human inoculum and pig inoculum were performed on separate days. Both *in vitro* fermentations were run in duplicate.

4.2.6 Analytical methods

4.2.6.1 Constituent monosaccharide composition

The constituent monosaccharide composition of the carbohydrates present in the substrates was analyzed as described previously *[10]*.

4.2.6.2 Short chain fatty acids and lactate

Acetate, propionate, butyrate and lactate were measured in triplicate using an HPLC system with conductivity detection equipped with an Aminex HPX-87H analytical column (7.8 mm × 300 mm; Bio-Rad Laboratories, Hercules, CA, USA). An anion micromembrane suppressor (AMMS-ICE300, Thermo Scientific, Waltham, MA, USA; 10 mM NH_4OH) was used to reduce the background conductivity. The column temperature was maintained at 45 °C and the flow rate was 0.6 mL/min. The injection volume was 50 µL. The mobile phase was composed of 1.6 mM heptafluorobutyric acid and elution was performed isocratically. An electrochemical detector (ED40, Thermo Scientific) was used in conductivity mode and set at a range of 200 µS. The data were processed using Empower software (Waters Corporation, Milford, MA, USA).

4.2.6.3 Oligosaccharide characterization

Hydrophilic interaction chromatography with mass spectrometry detection (HILIC-MS) was used to obtain information on the amount and type of oligosaccharides that were present at different time points during the *in vitro* fermentations. Chromatographic separation was performed on an Acquity UPLC BEH Amide column (1.7 µm, 2.1 mm × 150 mm; Waters Corporation) in combination with an Acquity UPLC BEH Amide VanGuard pre-column (1.7 µm, 2.1 mm × 5 mm; Waters Corporation). Analysis was performed as described previously *[17]*, using adapted gradient and MS-settings. The mobile phase was composed of (A) H_2O, (B) acetonitrile and (C) 200 mM ammonium formate with 2% (v/v) formic acid. The eluent profile was as follows: 0-30 min, linear from 15% A, 80% B and 5% C to 45% A, 50% B and 5% C; 30-32 min, linear to 75% A, 20% B and 5% C; 32-37 min, isocratic 75% A, 20% B and 5% C; 37-39 min, linear to 15% A, 80% B and 5% C; 39-45 min, isocratic 15% A, 80% B and 5% C. A Velos Pro ion trap MS was used (Thermo Scientific). MS detection was performed in negative mode with the ion source voltage set to −4.5 kV, the capillary voltage set to −5 V, a capillary temperature of 350 °C, a heated ESI source temperature of 225 °C, a sheath gas flow rate of 40 (arbitrary units), an auxiliary gas flow rate of 12 (arbitrary units) and a sweep gas flow rate of 2 (arbitrary units).

4.2.6.4 Molecular techniques

DNA from the *in vitro* fermentation samples was isolated using the AGOWA mag mini DNA isolation kit (AGOWA, Berlin, Germany), according to the manufacturer's instructions. Approximately 100 pg DNA template was used for PCR. Generation of the PCR amplicon library was performed by amplification of V5-V7 hypervariable region of the small subunit ribosomal DNA gene (16S rDNA). Amplification was performed using the forward primer 785F (5'-GGATTAGATACCCBRGTAGTC-3') and reverse primer 1175R (5'-ACGTCRTCCCCDCCTTCCTC-3'). The primers were fitted with the 454 Life Sciences Adapter A (forward primer) and B (reverse primer), fused to the 5' end of the 16S rDNA bacterial primer sequences. The forward primer also included a unique barcode. The amplification mix contained 2 units of Pfu Ultra II Fusion HS DNA polymerase (Stratagene, La Jolla, CA, USA) and 1x PfuUltra II reaction buffer (Stratagene), 200 µM dNTP PurePeak DNA polymerase mix (Pierce Nucleic Acid Technologies, Milwaukee, WI, USA) and 0.2 µM of each primer. After an initial denaturation (94 °C; 2 min), 30 PCR cycles were performed that consisted of denaturation (94 °C; 30 s), annealing (50 °C; 40 s), and extension (72 °C; 80 s). Samples with DNA recovery of equal or less than 10 pg/µL of DNA were cycled 35 times using the same protocol. Amplicons were size checked and quantified by gel electrophoresis and Quant-iT Picogreen dsDNA Assay (Invitrogen, Carlsbad, CA, USA) on an Infinite M200 (Tecan, Männedorf, Switzerland). Amplicons of the individual samples were pooled equimolar and purified from agarose gel by means of QIAquick Gel Extraction Kit Protocol (Qiagen, Hilden, Germany). The library was sequenced unidirectionally in the forward direction (A-adapter) in one run in the 454 GS-FLX-Titanium Sequencer (Roche, Branford, CT, USA) by Keygene (Wageningen, The Netherlands).

4.2.6.5 Sequence processing and analyses

FASTA-formatted sequences and corresponding quality scores were extracted from the .sff data file generated by the GS-FLX-Titanium sequencer using the GS Amplicon software package (Roche, Branford, CT). GS-FLX-titanium sequencing data was processed using modules implemented in the Mothur v. 1.20.0. software platform module *[18]*. Due to the unique barcodes, sequences were initially binned by sample of origin. For further downstream analyses, sequences were trimmed. In brief, barcodes, primer sequences and low quality data (containing ambiguous base calls (N) in the sequence, more than 8 homopolymers anywhere in the sequence, shorter than 50 nt after trimming with a window average below 35 or a length >500 or <200 bp) were removed. The data set was simplified by using the "unique.seqs" command to generate a non-redundant (unique) set of sequences. Unique sequences were aligned using the "align.seqs" command and an adaptation of the Bacterial SILVA SEED database as a template

(http://www.mothur.org/wiki/Alignment_database). Alignment was done using the RDP template. In order to ensure that comparable regions of the 16S rDNA gene were analyzed across all reads, sequences that started before the 2.5-percentile or ended after the 97.5-percentile in the alignment were filtered. Sequences were denoised using the "pre.cluster" command. This command applies a pseudo-single linkage algorithm with the goal of removing sequences that are likely due to pyrosequencing errors *[19]*. A total of 16814 potentially chimeric sequences were detected and removed using the "chimera.uchime" command *[20]*. High quality aligned sequences were classified by using the RDP-II naïve Bayesian Classifier database. Aligned sequences were clustered into operation taxonomic units (OTUs; defined by 97% similarity) and were calculated by the average linkage clustering commando method. Unclassified sequences were also grouped in OTUs and were represented with a random number to distinguish them from the different OTUs found within the same phyla.

4.3 Results and discussion

4.3.1 Constituent monosaccharide composition and structure of SBPOS and FOS

In order to characterize the substrates used in this study, their constituent monosaccharide composition was analyzed and oligosaccharide structures were identified. The constituent monosaccharide composition of SBPOS and FOS is given in Table 4.1. SBPOS were mainly composed of uronic acids (galacturonic and glucuronic acid in a ratio of 10:1), galactose, arabinose, rhamnose and, to a lesser extent, of glucose, xylose, mannose and fucose. Approximately 15% (w/w) of these saccharides were present as mono- or disaccharides (results not shown).

Table 4.1. Constituent monosaccharide composition and total sugar levels of substrates used for *in vitro* fermentations.

substrate	glc	fru	ara	gal	rha	xyl	man	fuc	galA	glcA	total
SBPOS	5	0	16	17	12	1	1	1	43	4	90
FOS	10	90	0	0	0	0	0	0	0	0	95

Sugar annotation: glc, glucose; fru, fructose; ara, arabinose; gal, galactose; rha, rhamnose; xyl, xylose; man, mannose; fuc, fucose; galA, galacturonic acid; glcA, glucuronic acid. Values are expressed as mol% of total sugar content of substrate samples. Total sugar content is expressed as w/w% of substrate samples.

The different oligosaccharide structures that were present in the SBPOS-mixture were identified using HILIC-MS (Figure 4.1). It was revealed that the SBPOS were composed of several classes of oligosaccharides, which mainly consisted of rhamnogalacturono-oligosaccharides, mostly containing a terminal galacturonic acid residue at both reducing and non-reducing end (Rha_nGalA_{n+1}), acetylated rhamnogalacturono-oligosaccharides ($Rha_nGalA_{n+1}Ac_x$), partially methyl esterified and/or acetylated homogalacturono-oligosaccharides ($GalA_nAc_xMe_y$), and partially methyl esterified and/or acetylated homogalacturono-oligosaccharides containing an unsaturated galacturonic acid residue at the non-reducing end ($uGalAGalA_nAc_xMe_y$). Precise structural details of these classes of pectic oligosaccharides have been described elsewhere *[10]*. These special classes of oligosaccharides are distinct from the classes of sugar beet pectic oligosaccharides that have been used for *in vitro* fermentation studies previously *[4-6]*. Besides these classes of oligosaccharides, smaller amounts of arabino- and galacto-oligosaccharides were present. The average degree of polymerization of the SBPOS was around 5.

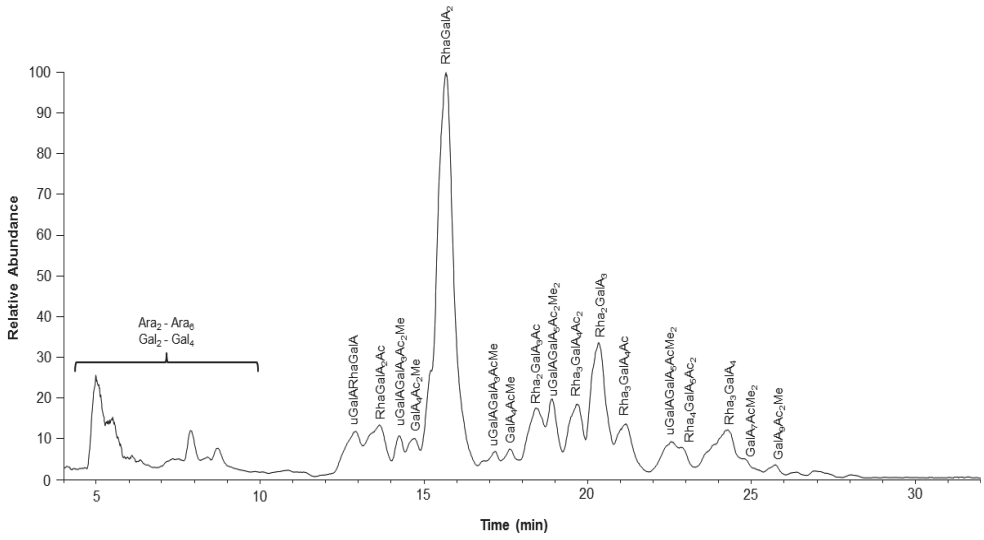

Figure 4.1. Hydrophilic interaction chromatography (HILIC) elution pattern of sugar beet pectic oligosaccharides (SBPOS). Peak annotation: Ara, arabinose; Gal, galactose; GalA, galacturonic acid; uGalA, unsaturated galacturonic acid; Rha, rhamnose; Ac, acetyl group; Me, methyl ester.

FOS were composed of fructose and glucose residues in a ratio of 9:1. Approximately 8% (w/w) of the fructose and glucose in FOS was present as mono- or disaccharides (results not shown). FOS are obtained by partial enzymatic hydrolysis of inulin and are composed of a mixture of linear oligofructose chains, which can be terminated by a glucose residue. The fructose molecules are linked to each other by β-(2,1)-glycosidic bonds. The average degree of polymerization of FOS was 4 to 5 (according to supplier's specification).

4.3.2 Changes in pH

The pH was measured to monitor the course of the *in vitro* fermentations. Table 4.2 shows the pH after 0, 3, 6, 9, 12, 24 and 48 h of *in vitro* fermentation. For both SBPOS and FOS the pH initially decreased. For SPBO, the pH increased again after 12 h (human fecal inoculum) and 6 h (pig fecal inoculum). For FOS, the pH increased again after 9 h (in case of both human and pig fecal inoculum). The decrease in pH is the result of the initially high carbohydrate availability, which results in saccharolytic fermentation and subsequent formation of SCFA and lactate, thereby lowering the pH. The decrease in pH was largest for FOS. It is likely that this was caused by the formation of lactic acid, which has a lower pK_a (3.73) compared to acetic, propionic and butyric acid (4.76, 4.87 and 4.82, respectively). The observed increase in pH after a certain time point may be partly due to a shift to a more proteolytic fermentation *[21]*. The protein degradation products (e.g. ammonia) will increase the pH. For FOS, the decrease in lactate concentration (e.g. after 12-24 h) will also have contributed to the observed pH increase during the end of the *in vitro* fermentations. The gradual increase of the pH of the control samples (medium without added substrate) is most likely caused by early protein degradation due to lack of available carbohydrates *[21]*. For SBPOS, the pH at t=0 was lower compared to FOS and the control, due to the acidic nature of SBPOS.

4.3.3 Formation of SCFA and lactate

The SCFA and lactate formation during *in vitro* fermentation is often used as an indicator of fiber fermentability. In our study, an increase in acetate, propionate, butyrate and lactate was measured (Tables 4.2A and 4.2B). Other metabolites, such as valerate and succinate, were also analyzed and were present only in minor amounts (results not shown). The total SCFA content at the end of the fermentation was higher for SBPOS than for FOS, both when human and pig fecal inocula were used (Tables 4.2A and 4.2B). Substantial amounts of lactate were produced during the first stage of fermentation of FOS, both by human and pig fecal microbiota. The ratios acetate:propionate:butyrate:lactate for SBPOS were generally different

compared to the ratios for FOS. Acetate and propionate were mainly produced during *in vitro* fermentation of SBPOS, while for FOS, lactate and acetate were the most dominant SCFA. Total SCFA concentrations were higher when using human fecal inoculum compared to pig fecal inoculum, both for SBPOS and FOS, which is in accordance with previous work *[12]*. However, more lactate was produced by pig fecal microbiota. This was especially the case for FOS, but also for SBPOS. The proportion of butyrate considerably increased after 24-48 h of fermentation using human fecal inoculum and after 12-24 h using pig fecal inoculum. An increased butyrate production and subsequently higher butyrate proportion during the second stage of *in vitro* fermentation of pectic oligosaccharides and FOS was reported previously *[22, 23]*. For pig fecal microbiota, lactate was the major metabolite during the first 12 h of fermentation of FOS. The formation of lactate during *in vitro* fermentation of FOS is in accordance with previous studies *[12, 24]*. Lactate is an intermediary product of carbohydrate fermentation and can be converted into acetate, propionate and butyrate by common intestinal bacteria *[25]*. This explains the decrease of lactate and the large increase of butyrate during the second stage of fermentation of FOS (i.e. between 24 h and 48 h for human fecal inoculum and between 12 and 24 h for pig fecal inoculum). Part of the butyrate may also have been formed from acetate as butyrate producing bacteria can be net utilizers of acetate *[26]*.

4.3.4 Oligosaccharide degradation

The degradation of SBPOS during *in vitro* fermentations was monitored by HILIC-MS and is shown in Figure 4.2. The SBPOS were utilized after 9 h of *in vitro* fermentation by human fecal microbiota (Figure 4.2A). The increase in pH after this time corroborates that the microbiota switched to proteolytic fermentation, as all available carbohydrates were used up. Figure 4.2B illustrates that the degradation pattern of SBPOS during *in vitro* fermentation using pig fecal inoculum is quite different compared to the human fecal inoculum. Using pig fecal inoculum, most of the SBPOS were still clearly detectable after 12 h and some of the SBPOS were even detectable after 24 h of *in vitro* fermentation. Previously, it was demonstrated that pig fecal microbiota had limited ability to ferment citrus and soy pectin compared to human fecal microbiota *[12]*. Therefore, it can be expected that SBPOS are also degraded slower by pig fecal microbiota compared to human fecal microbiota.

Table 4.2A. pH, short chain fatty acids (SCFA) and lactate formation during *in vitro* fermentation of sugar beet pectic oligosaccharides (SBPOS) and fructo-oligosaccharides (FOS) using human fecal inoculum.

substrate	time (h)	human fecal inoculum					
		pH	acetate (mmol/g)	propionate (mmol/g)	butyrate (mmol/g)	total SCFA (mmol/g)	lactate (mmol/g)
SBPOS	0	6.05 (0.00)	0.23 (0.05)	0.05 (0.01)	0.05 (0.01)	0.33 (0.05)	0.03 (0.01)
	3	6.12 (0.01)	1.34 (0.03)	0.34 (0.02)	0.24 (0.01)	1.92 (0.04)	0.12 (0.01)
	6	6.11 (0.01)	3.70 (0.19)	1.04 (0.02)	0.53 (0.02)	5.28 (0.22)	0.08 (0.01)
	9	5.93 (0.01)	6.60 (0.11)	2.20 (0.09)	0.86 (0.03)	9.66 (0.11)	0.02 (0.01)
	12	5.90 (0.01)	8.08 (0.11)	2.90 (0.09)	1.03 (0.03)	12.01 (0.18)	0.02 (0.01)
	24	5.94 (0.01)	9.81 (0.19)	4.00 (0.28)	1.81 (0.07)	15.61 (0.30)	0.02 (0.01)
	48	6.16 (0.01)	11.60 (0.11)	4.95 (0.15)	3.11 (0.07)	19.66 (0.24)	0.02 (0.01)
FOS	0	6.16 (0.01)	0.25 (0.05)	0.05 (0.01)	0.04 (0.01)	0.34 (0.06)	0.04 (0.01)
	3	6.13 (0.00)	1.17 (0.02)	0.21 (0.01)	0.22 (0.01)	1.60 (0.03)	0.22 (0.01)
	6	5.14 (0.01)	4.21 (1.15)	0.47 (0.09)	0.70 (0.20)	5.38 (1.44)	1.91 (0.55)
	9	4.91 (0.01)	6.78 (0.27)	0.64 (0.08)	1.15 (0.07)	8.58 (0.34)	3.39 (0.12)
	12	4.95 (0.03)	7.25 (0.20)	0.79 (0.07)	1.20 (0.11)	9.23 (0.36)	3.41 (0.30)
	24	5.06 (0.02)	7.42 (0.10)	0.83 (0.03)	1.23 (0.07)	9.49 (0.09)	3.51 (0.12)
	48	5.54 (0.01)	4.56 (0.18)	0.87 (0.05)	4.86 (0.07)	10.29 (0.18)	0.04 (0.01)
Control	0	6.15 (0.00)	0.19 (0.03)	0.04 (0.01)	0.03 (0.01)	0.26 (0.02)	0.03 (0.01)
	9	6.47 (0.01)	2.28 (0.25)	0.62 (0.03)	0.64 (0.07)	3.54 (0.35)	0.06 (0.01)
	24	6.61 (0.01)	4.25 (0.08)	1.44 (0.08)	1.25 (0.11)	6.94 (0.11)	0.01 (0.01)

Values are means of triplicate measurements with standard deviation in parentheses. SCFA and lactate results are expressed as mmol/g organic matter.

Table 4.2B. pH, short chain fatty acids (SCFA) and lactate formation during *in vitro* fermentation of sugar beet pectic oligosaccharides (SBPOS) and fructo-oligosaccharides (FOS) using pig fecal inoculum.

substrate	time (h)	pig fecal inoculum					
		pH	acetate (mmol/g)	propionate (mmol/g)	butyrate (mmol/g)	total SCFA (mmol/g)	lactate (mmol/g)
SBPOS	0	5.97 (0.02)	0.16 (0.01)	< 0.01 (-)	< 0.01 (-)	0.16 (0.01)	0.06 (0.01)
	3	5.87 (0.00)	0.63 (0.02)	0.13 (0.01)	0.03 (0.01)	0.79 (0.03)	0.47 (0.04)
	6	5.82 (0.03)	1.36 (0.08)	0.30 (0.03)	0.03 (0.03)	1.69 (0.13)	0.57 (0.07)
	9	5.98 (0.01)	1.97 (0.11)	0.70 (0.04)	0.19 (0.01)	2.86 (0.06)	0.35 (0.04)
	12	6.10 (0.01)	2.90 (0.21)	0.96 (0.03)	0.50 (0.02)	4.36 (0.21)	< 0.01 (-)
	24	6.14 (0.01)	5.30 (0.11)	2.40 (0.15)	2.23 (0.07)	9.93 (0.32)	< 0.01 (-)
	48	6.34 (0.01)	8.14 (0.56)	3.70 (0.23)	3.24 (0.19)	15.08 (0.97)	< 0.01 (-)
FOS	0	6.19 (0.02)	0.13 (0.01)	< 0.01 (-)	< 0.01 (-)	0.13 (0.01)	0.05 (0.01)
	3	5.99 (0.02)	0.48 (0.03)	0.15 (0.02)	< 0.01 (-)	0.63 (0.05)	0.96 (0.03)
	6	5.66 (0.01)	1.38 (0.02)	0.35 (0.02)	< 0.10 (-)	1.73 (0.01)	2.53 (0.07)
	9	4.59 (0.02)	2.98 (0.18)	0.69 (0.07)	0.08 (0.01)	3.74 (0.25)	7.52 (0.59)
	12	4.75 (0.02)	4.15 (0.27)	1.89 (0.08)	0.34 (0.02)	6.38 (0.35)	5.69 (0.18)
	24	5.67 (0.00)	2.05 (0.10)	1.47 (0.08)	3.15 (0.14)	6.66 (0.20)	< 0.01 (-)
	48	6.22 (0.01)	3.62 (0.10)	2.18 (0.16)	3.90 (0.14)	9.70 (0.09)	< 0.01 (-)
Control	0	6.20 (0.01)	0.19 (0.02)	< 0.01 (-)	< 0.01 (-)	0.19 (0.02)	0.03 (0.01)
	9	6.32 (0.01)	1.09 (0.01)	0.53 (0.02)	0.12 (0.01)	1.74 (0.03)	< 0.01 (-)
	24	6.68 (0.02)	3.66 (0.17)	1.18 (0.01)	1.44 (0.07)	6.28 (0.12)	< 0.01 (-)

Values are means of triplicate measurements with standard deviation in parentheses. SCFA and lactate results are expressed as mmol/g organic matter.

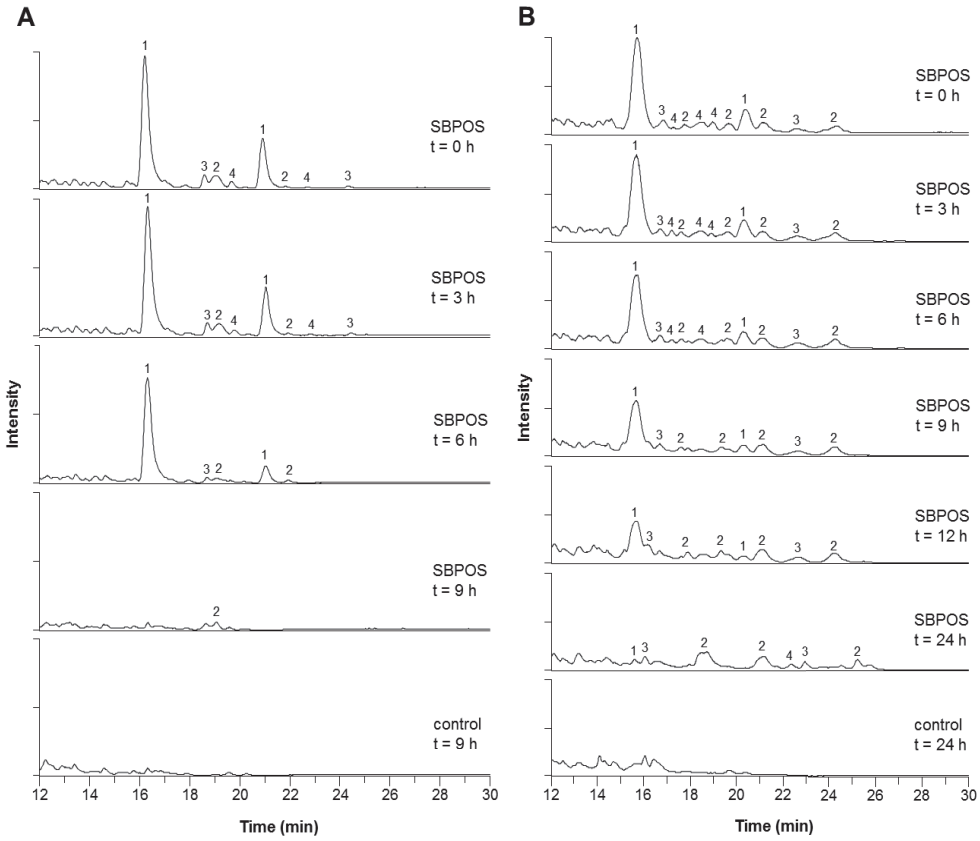

Figure 4.2. Hydrophilic interaction chromatography (HILIC) elution patterns obtained after several time points during *in vitro* fermentation of sugar beet pectic oligosaccharides (SBPOS) using human (A) or pig (B) fecal inoculum. HILIC elution patterns obtained after 9 h (human fecal inoculum) and 24 h (pig fecal inoculum) of *in vitro* fermentation of the control sample are presented to show which peaks correspond to the medium components. Peak annotation: 1, Rha_nGalA_{n+1}; 2, $Rha_nGalA_{n+1}Ac_x$; 3, $GalA_nAc_xMe_y$; 4, $uGalAGalA_nAc_xMe_y$.

HILIC-MS analysis also enabled to plot the degradation rate of the different classes of oligosaccharides that were present after different fermentation times. Figure 4.3 shows the results for the first 24 h of *in vitro* fermentation. Using human fecal inoculum, the degradation rate of the rhamnogalacturono-oligosaccharides (Rha_nGalA_{n+1} and $Rha_nGalA_{n+1}Ac_x$) and partially methyl esterified and/or acetylated homogalacturono-oligosaccharides ($GalA_nAc_xMe_y$) tended to be a bit slower during

the first 6 h of *in vitro* fermentation compared to the other classes of oligosaccharides (Figure 4.3A). Nevertheless, most of the oligosaccharides were fermented after 9 h of fermentation. For comparison, the degradation of FOS during the first 6 h of *in vitro* fermentation appeared to be faster than most of the SBPOS-classes (Figure 4.3A).

Figure 4.3B illustrates that the class $Rha_nGalA_{n+1}Ac_x$ was slower degraded by pig fecal microbiota than the other three SBPO-classes. The degradation rate of all classes of SBPOS by pig fecal microbiota was lower compared to human fecal microbiota. $Rha_nGalA_{n+1}Ac_x$ showed to be the most recalcitrant class of oligosaccharides, as approximately half of the initial amount was still detectable after 24 h. For comparison, FOS were completely utilized after 9 h of *in vitro* fermentation. Nevertheless, FOS was fermented slower by pig fecal microbiota compared to human fecal microbiota.

It was demonstrated previously that the degree of methyl esterification of pectic oligosaccharides influences the degradation rate *in vitro [11]*. Our study shows that acetylation of rhamnogalacturono-oligosaccharides also hinders the degradation by pig fecal microbiota, but not by human fecal microbiota. The latter differs from results on xylo-oligosaccharides *[27]*, where acetylated xylo-oligosaccharides were degraded more slowly by human fecal microbiota compared to nonsubstituted xylo-oligosaccharides.

All classes of SBPOS were used up after 48 h (data not shown), thereby showing that both human and pig fecal microbiota possess the appropriate enzymes to fully degrade the SBPOS. The slower degradation of pectin compared to FOS by pig fecal microbiota is in accordance with previous findings *[12]*. Most likely, pig fecal microbiota were not very well adapted to the utilization of pectic oligosaccharides, since the diet of the pigs used in our study was mainly maize- and wheat-based, therefore containing low amounts of pectin *[28]*. Human fecal microbiota are much more adapted to pectic oligosaccharides since the human diet usually contains pectin-rich fruits or vegetables *[29]*.

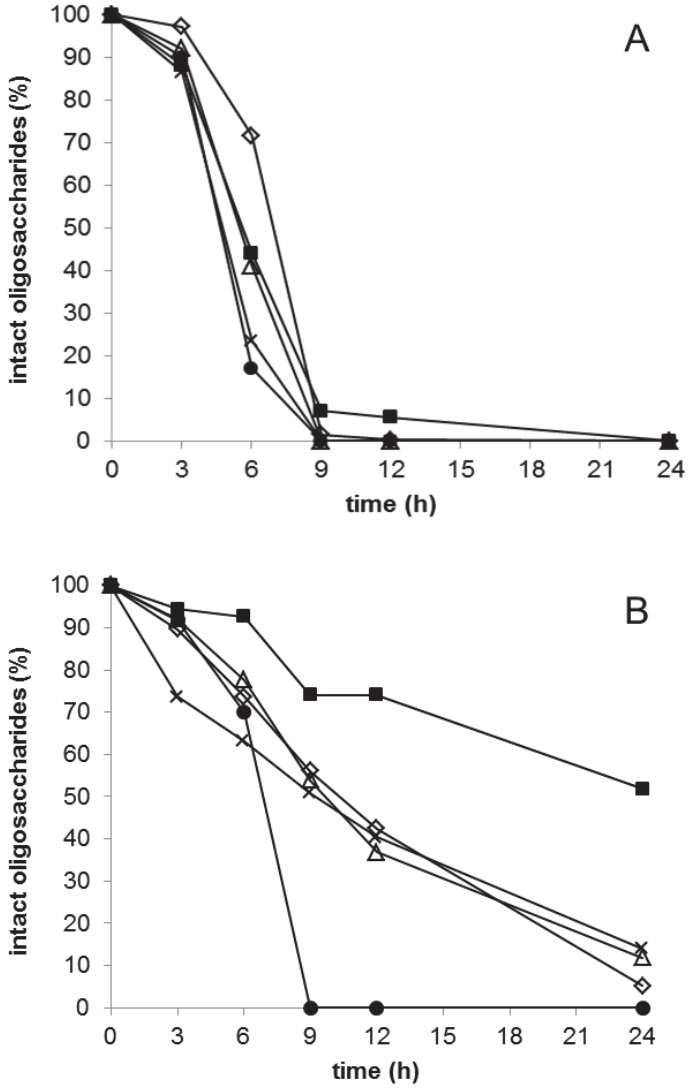

Figure 4.3. Degradation rate, expressed as decrease in percentage of the initial content of different classes of oligosaccharides during 24 h of *in vitro* fermentation by human (A) or pig (B) fecal inoculum. Legend: ◇, Rha_nGalA_{n+1}; ■, $Rha_nGalA_{n+1}Ac_x$; △, $GalA_nAc_xMe_y$; ×, $uGalAGalA_nAc_xMe_y$; ●, FOS.

4.3.5 Changes in microbiota composition

Figure 4.4 shows the human fecal microbiota composition on genus level at different time points of *in vitro* fermentation of SBPOS compared to FOS and the control. The initial microbiota composition was rather similar for the different fermentations, as expected.

Bacteroides were mainly stimulated during *in vitro* fermentation of SBPOS (Figure 4.4). No bifidogenic effect was noticed for SBPOS, since no increase in the population of *Bifidobacterium* was observed during *in vitro* fermentation of SBPOS. By contrast, a considerable increase in the population of *Bifidobacterium* was observed after 5 and 9 h of *in vitro* fermentation of FOS. Our findings are in agreement with previous studies *[13, 30]*, showing the limited bifidogenic effect of pectic oligosaccharides compared to FOS. It was also described previously that *Bacteroides* spp. were able to metabolize rhamnogalacturono-oligosaccharides, whereas *Bifidobacterium* strains were not able to metabolize galacturono-oligosaccharides *[31]*.

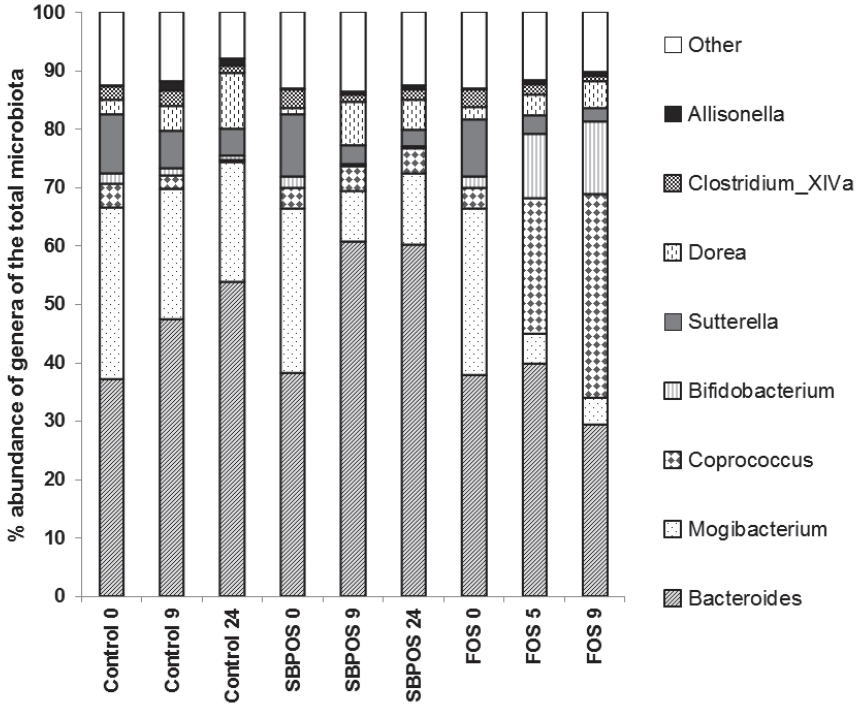

Figure 4.4. Human fecal microbiota composition (genus level) during *in vitro* fermentations of sugar beet pectic oligosaccharides (SBPOS) and fructo-oligosaccharides (FOS).

In order to highlight other interesting changes in bacterial populations, the changes in microbial genera composition were calculated after 9 and 24 h of *in vitro* fermentation, compared to the control incubation (Table 4.3). It is shown that *Bifidobacterium* spp. were relatively decreased when SBPOS was added as a substrate. The growth of several other groups of bacteria was increased during *in vitro* fermentation of SBPOS. *Blautia* spp. increased after 9 and 24 h of *in vitro* fermentation (6 and 33-fold, resp.). These bacteria were found to be reduced in patients with colorectal cancer *[32]*. Some species, such as *Blautia hydrogenotrophica*, have been shown to boost the efficiency of the fermentation of dietary carbohydrates *[33]*. A 10-fold increase in *Coprococcus* spp. was also noticed after 24 h of *in vitro* fermentation of SBPOS. As a comparison, the increase in *Coprococcus* spp. was even higher for FOS (i.e. 18-fold increase after 9 h of *in vitro* fermentation). *Coprococcus* spp. have been recognized previously to produce high amounts of butyrate *in vitro [34]*. Furthermore, it has been hypothesized that when the growth of this group of bacteria together with *Dorea* spp. is suppressed, it may lead to the flourishing of other groups, such as clostridia, which are linked to inflammation *[35]*. In addition, an increase in *Butyricicoccus* spp. was observed. It has been described previously that patients with inflammatory bowel disease had lower numbers of *Butyricicoccus* spp. in their stools than healthy subjects and oral administration of *Butyricicoccus pullicaecorum* improved the epithelial barrier function *[36]*. *Faecalibacterium* spp. also increased and members of this group are known by their anti-inflammatory properties. Their decrease is associated with ulcerative colitis *[37]*.

Besides the stimulation of certain groups of bacteria that can be associated with host health, a decrease of several groups of bacteria that may have adverse effects on host health and well-being was also noticeable. *Sutterella* spp. decreased after 9 and 24 h of *in vitro* fermentation of SBPOS. Members of this group have been isolated from individuals suffering from ulcerative colitis and Crohn's disease and are abundant in children with gastrointestinal dysfunction and autism *[38]*. *Alistipes* spp. and *Allisonella* spp. also decreased after 9 and 24 h of *in vitro* fermentation of SBPOS. Several species of *Alistipes* are mostly correlated with frequency of abdominal pain in patients with irritable bowel syndrome *[39]*. Proportions of *Allisonella* spp. are high in patients with irritable bowel syndrome with diarrhea-constipation episodes *[40]*.

Table 4.3. Fold changes in microbiotal genera after 9 h and 24 h of *in vitro* fermentation of sugar beet pectic oligosaccharides (SBPOS) and 9 h of *in vitro* fermentation of fructo-oligosaccharides (FOS) using human fecal inoculum.[a]

Genus	SBPOS		FOS
	t = 9 h	t = 24 h	t = 9 h
Bifidobacterium	0.17	0.30	8.17
Slackia	0.12	0.76	0.51
Parabacteroides	1.06	2.36	0.85
Paraprevotella	1.47	2.15	0.57
Prevotella	0.51	0.45	0.44
Alistipes	0.24	0.37	0.21
Bacteroides	1.24	1.09	0.61
Sutterella	0.41	0.65	0.37
Bilophila	0.11	0.06	0.35
Escherichia_Shigella	0.39	0.33	0.66
Clostridium_sensu_stricto	0.55	0.29	0.06
Mogibacterium	0.41	0.62	0.21
Blautia	6.89	33.99	3.09
Clostridium_XlVa	0.36	1.06	0.32
Coprococcus	2.22	10.56	18.40
Dorea	2.25	0.70	1.28
Lachnospiracea_incertae_sedis	7.52	12.04	6.29
Butyricicoccus	2.67	7.11	6.68
Faecalibacterium	11.47	14.89	1.58
Oscillibacter	0.81	0.55	0.81
Catenibacterium	0.63	3.54	12.99
Erysipelotrichaceae_incertae_sedis	1.66	1.90	5.44
Phascolarctobacterium	4.20	1.13	0.47
Allisonella	0.24	0.41	0.39
Dialister	1.94	4.96	3.64

[a] For each substrate, the ratio between a sampling time point and t = 0 was calculated (e.g. t9/t0 and t24/t0). The ratio for this value and the corresponding control was then determined to obtain fold changes. A value equal to 1 indicates no change, a value > 1 indicates an increase and a value < 1 indicates a decrease of the respective microbiotal genera.

4.4 Conclusions

This study provides new information on the *in vitro* fermentation characteristics of SBPOS. It was shown that SBPOS can be completely fermented by human and pig fecal microbiota, thereby producing butyrate, yet mainly acetate and propionate. The degradation rate of SBPOS by pig fecal microbiota was lower compared to human fecal microbiota. In general, rhamnogalacturono-oligosaccharides were more slowly degraded than homogalacturono-oligosaccharides. Acetylation of rhamnogalacturono-oligosaccharides lowered the degradation rate by pig fecal microbiota, but not by human fecal microbiota. A classic prebiotic effect could not be demonstrated for SBPOS, since no increase in bifidobacteria or lactobacilli was observed. However, several other potentially beneficial modifications in the microbiota composition were observed during *in vitro* fermentation of SBPOS using human fecal inoculum. Further *in vivo* work is needed to ultimately confirm these specific prebiotic properties of SBPOS. Since SBPOS are produced as a sidestream of the production of monosaccharides from sugar beet pulp, they are available in large quantities. This makes it feasible to conduct future *in vivo* experiments with SBPOS.

Acknowledgements

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Chapter 5

Identification of novel isomeric pectic oligosaccharides using hydrophilic interaction chromatography coupled to traveling-wave ion mobility mass spectrometry

Based on:

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Abstract

Separation and characterization of complex mixtures of pectic oligosaccharides still remains challenging and often requires the use of multiple analytical techniques, especially when isomeric structures are present. In this work, it is demonstrated that the coupling of hydrophilic interaction chromatography (HILIC) to traveling-wave ion mobility mass spectrometry (TWIMS) enabled the simultaneous separation and characterization of complex mixtures of various isomeric pectic oligosaccharides. Labeling of oligosaccharides with 3-aminoquinoline (3-AQ) improved MS-ionization efficiency of the oligosaccharides and reduced the complexity of the product ion mass spectra, without losing resolution of the HILIC separation. In addition, labeling enabled quantification of oligosaccharides on molar basis using in-line fluorescence detection. Isomeric structures were distinguished using TWIMS. The developed method was used to characterize series of isomeric sugar beet rhamnogalacturonan I derived oligosaccharides carrying a glucuronic acid substituent. Thereby, some novel structural features were identified for the first time: Glucuronic acid was attached to *O*-3 or to *O*-2 of galacturonic acid residues and a single galacturonic acid residue within an oligomer could contain both an acetyl group and a glucuronic acid substituent.

5.1 Introduction

Plant cell walls are responsible for the stability, rigidity and flexibility of plant cells. They are predominantly composed of the polysaccharides cellulose, hemicellulose and pectin, which form a complex network *[1, 2]*. Pectin is regarded as a key component for the architecture of dicotyledonous plant cell walls due to its multiple interaction properties. It is a highly complex polysaccharide as it can be composed of 17 different monosaccharides and contains more than 20 different linkages *[3]*. It consists of several structural elements: homogalacturonan, xylogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II. The amount, precise structure and length of each structural element largely depends on the plant origin, cell type and stage of cellular development *[4]*.

Upon enzymatic degradation of pectin, complex mixtures of oligosaccharides and monosaccharides are formed. Separation and characterization of these mixtures are necessary to obtain information about the structural characteristics of the degradation products. Nowadays, different chromatographic and mass spectrometry techniques are available for the separation and characterization of complex mixtures of pectic oligosaccharides *[5-7]*. In recent years, hydrophilic interaction chromatography (HILIC) coupled to mass spectrometry (MS) has proven to be a valuable technique for detailed separation and characterization of a wide range of pectic oligosaccharides *[8, 9]*. Using HILIC, important structural features of the pectic oligosaccharides (e.g. methyl esters and acetyl groups) are retained during analysis *[10]*. However, isomeric oligosaccharides, i.e. structurally different oligosaccharides with identical composition, are mostly not separated by HILIC *[11]*. Pectin digested with backbone degrading enzymes can contain many isomeric oligosaccharides, because of the variation in distribution of different substituents along the pectin backbone. Therefore, the use of HILIC-MS is limited for obtaining information about the presence, relative quantity and structure of isomeric oligosaccharides in pectin digests.

Ion mobility MS separates gas-phase ions based on their three-dimensional shape as reflected by their mobility in an inert buffer gas. Therefore, this type of MS can potentially distinguish isomers with identical *m/z* values but different structures or configurations *[12]*. Ion mobility MS has demonstrated to be valuable for the rapid identification of mixtures of isomeric *N*-glycan oligosaccharides released from glycoproteins *[13, 14]* or isomeric oligosaccharides derived from dextran and pullulan *[15]*. Recently, a rapid traveling-wave ion mobility MS (TWIMS) method was developed for the identification of various isomeric oligogalacturonic acids in mixtures of oligosaccharides with various degrees of polymerization and levels of methyl esterification, using 3-aminoquinoline (3-AQ) as a labeling agent. Using this 3-AQ-TWIMS method, the precise distribution of methyl esters for several isomeric

oligosaccharides was determined *[16]*. Unfortunately, the sensitivity of the TWIMS instrument that was used (i.e. Synapt G1 HDMS) was insufficient to couple TWIMS to HILIC for separating complex oligosaccharide mixtures prior to TWIMS to enable rapid analysis of these mixtures. It has been shown *[17]* that the combination of HILIC with a new generation TWIMS with a higher sensitivity (i.e. Synapt G2-S HDMS) was suitable for the confident and rapid distinction of *N*-glycan structures within a defined mixture. However, little has been reported on the potential of this analytical tool for plant derived oligosaccharides. Therefore, the present study aims at coupling HILIC to a new generation TWIMS to be able to separate and characterize complex mixtures of isomeric pectic oligosaccharides.

5.2 Materials and methods

5.2.1 Materials

Acetonitrile, ammonium formate, formic acid and water used to prepare the chromatographic eluents were of ULC/MS quality and were purchased from Biosolve (Valkenswaard, The Netherlands). All other chemicals used in this study were at least of analytical grade. Mixtures of partially methyl esterified α-(1,4)-linked galacturonic acid oligosaccharides were obtained after digestion of a 30% methyl esterified (DM30) lemon pectin with endopolygalacturonase from *Aspergillus niger* as described previously *[16]*. Mixtures of partially saturated and unsaturated methyl esterified α-(1,4)-linked galacturonic acid oligosaccharides were obtained after digestion of a 56% methyl esterified (DM56) lemon pectin with endopolygalacturonase and pectin lyase (both from *Aspergillus niger*) as described previously *[9]*. Mixtures of recalcitrant sugar beet pectin derived oligosaccharides were obtained from Cosun Food Technology Centre (Roosendaal, The Netherlands). This mixture was prepared by exhaustive enzymatic hydrolysis of sugar beet pulp (48 h at 45 °C; using an overdose of a combination of pectinases from *Aspergillus aculeatus* and *Aspergillus niger* as described previously *[8]*). All samples were stored at −20 °C until further use.

5.2.2 Fluorescent labeling of oligosaccharides

Derivatization of oligosaccharides with 3-AQ was carried out as described previously *[16]*. Samples were diluted with 50:50 (v/v) acetonitrile:water to a concentration of 1 mg/mL for HILIC-TWIMS analysis.

5.2.3 Monosaccharide composition of recalcitrant sugar beet pectin derived oligosaccharides

Methanolysis was performed using a slightly modified version of the procedure described previously *[18]*. In brief, 25 mg of dried oligosaccharides were mixed with 25 mL of 3 M anhydrous methanolic HCl followed by heating for 3 h at 95 °C. After cooling under a pressurized air flow, the sample was transferred to a round bottom flask, followed by evaporation using a rotary vacuum evaporator. The dried sample was submitted to hydrolysis by adding 25 mL 2 M trifluoroacetic acid and heating for 1 h at 115 °C. After cooling, the sample was neutralized by adding a few drops of 20% (w/w) NaOH-solution, dried, rediluted in 50 mL of distilled water and subsequently analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described previously *[8]*.

5.2.4 HILIC-FLR-TWIMS

An Acquity UPLC H-Class Bio System (Waters Corporation, Milford, MA, USA), equipped with an Acquity UPLC FLR detector was coupled to a Synapt G2Si high definition MS (Waters), equipped with a Z-spray ESI-source and traveling-wave ion mobility MS (TWIMS). Chromatographic separation was performed on an Acquity UPLC BEH Amide column (1.7 µm, 2.1 mm × 150 mm; Waters) in combination with an Acquity UPLC BEH Amide VanGuard precolumn (1.7 µm, 2.1 mm × 5 mm; Waters). Elution was performed at a flow rate of 300 µL/min and a column oven temperature of 35 °C. The injection volume was set to 1 µL. The mobile phase was composed of (A) water, (B) acetonitrile, and (C) 200 mM ammonium formate in 2% (v/v) formic acid. The eluent profile was as follows: 0-30 min, linear from 15% A, 80% B, and 5% C to 45% A and 50% B; 30-32 min, isocratic 45% A and 50% B; 32-34 min, linear to 15% A and 80% B; and 34-40 min, isocratic 15% A and 80% B. Fluorescence detection was performed in 2D-mode with a data rate of 1 point/s and a PMT gain of 1. The λ_{ex} was set to 355 nm and the λ_{em} to 420 nm, being the optimal excitation and emission wavelengths for 3-AQ *[19]*. The sample flow during the first 5 min of elution was directed to the waste to prevent salts and excess labeling reagent entering the TWIMS. Mass measurements were performed in positive and/or negative mode with the standard resolution settings. The ion source conditions were: Capillary voltage 3.0 kV, sampling cone voltage 45 V, ion source temperature 120 °C, desolvation gas temperature 600 °C, cone gas flow 100 L/h, and desolvation gas flow 800 L/h. For ion mobility measurements, helium gas was introduced into the entrance of the mobility cell and nitrogen was used as a drift gas. For ion mobility separation, IMS wave velocity and pulse height were set at 300 m/s and 40 V, respectively. Transfer collision energy was set to 50 V, enabling fragmentation of the ions after the ion mobility cell and before entering the time-of-

flight (TOF) MS. The instrument was calibrated in the mass range of 100-2000 Da using a sodium iodide solution (2 µg/µL in 50:50 (v/v) 2-propanol:water). Data were processed using MassLynx version 4.1 and DriftScope version 2.7 software (Waters). The fragmentation pattern was indicated following the nomenclature according to Domon and Costello *[20]*.

5.2.5 Calculation of theoretical collision cross section (CCS)

Computationally estimated CCSs were obtained after drawing the 3D-structures of the molecules using ChemBioDraw Ultra version 13.0 software (CambridgeSoft, Cambridge, MA, USA), calculation of the optimal geometry of the molecules using Avogadro software *[21]* and subsequent calculation of the theoretical CCS (Ω_{th}) using the CCS calculator within the DriftScope software (Waters).

5.3 Results and discussion

5.3.1 Effect of fluorescent labeling of oligosaccharides

The oligosaccharide mixtures used in this study were labeled with 3-AQ using a relatively simple procedure *[16]*. It enables sensitive analysis of oligosaccharides using HILIC with fluorescence (FLR) and MS detection and reduces the complexity of product ion mass spectra *[19, 22]*. First, the effect of 3-AQ labeling on the HILIC-separation and the MS ionization efficiency of pectic oligosaccharides was investigated. To this end, a known *[16]* endopolygalacturonase-digest of a 30% methylesterified (DM30) lemon pectin was analyzed using HILIC-MS before and after labeling with 3-AQ. Figures 5.1A and 5.1B show the HILIC-MS base peak chromatograms of the unlabeled and the 3-AQ-labeled DM30-pectin digests, respectively. Peaks were annotated with the corresponding x^y-oligosaccharide, based on the masses detected, where x represents the degree of polymerization and y represents the number of methyl esters. From Figure 5.1 it is clear that the elution pattern of partially methyl esterified galacturonic acid oligosaccharides using HILIC is dominated by the net charge of the oligosaccharides, which has also been shown previously *[10]*. For example, the oligosaccharides with a net charge of −3 (e.g. 3^0, 4^1, 5^2, 6^3) elute after the oligosaccharides with a net charge of −2 (e.g. 2^0, 3^1, 4^2). Labeling of oligosaccharides with 3-AQ resulted in decreased polarity of the oligosaccharides and, therefore, in decreased retention on the HILIC-column. Nevertheless, the order of elution was not changed and the resolution still remained adequate. Next to the decreased retention times, the relative MS signal intensities of the various oligosaccharides were different after 3-AQ-labeling. Figure 5.1B shows a larger relative abundance in the HILIC-MS base peak chromatogram

of several oligosaccharides (e.g. 2^0, 4^0) compared to Figure 5.1A. Furthermore, oligosaccharides with a net charge of −5 (e.g. 6^1, 7^2, 8^3) are clearly visible in Figure 5.1B, while they are hardly detectable in Figure 5.1A. It has been reported previously that labeling of oligosaccharides improves their ionization efficiency using ESI-MS *[23]*. The extent of this effect, as observed in Figure 5.1, is not the same for all oligosaccharides.

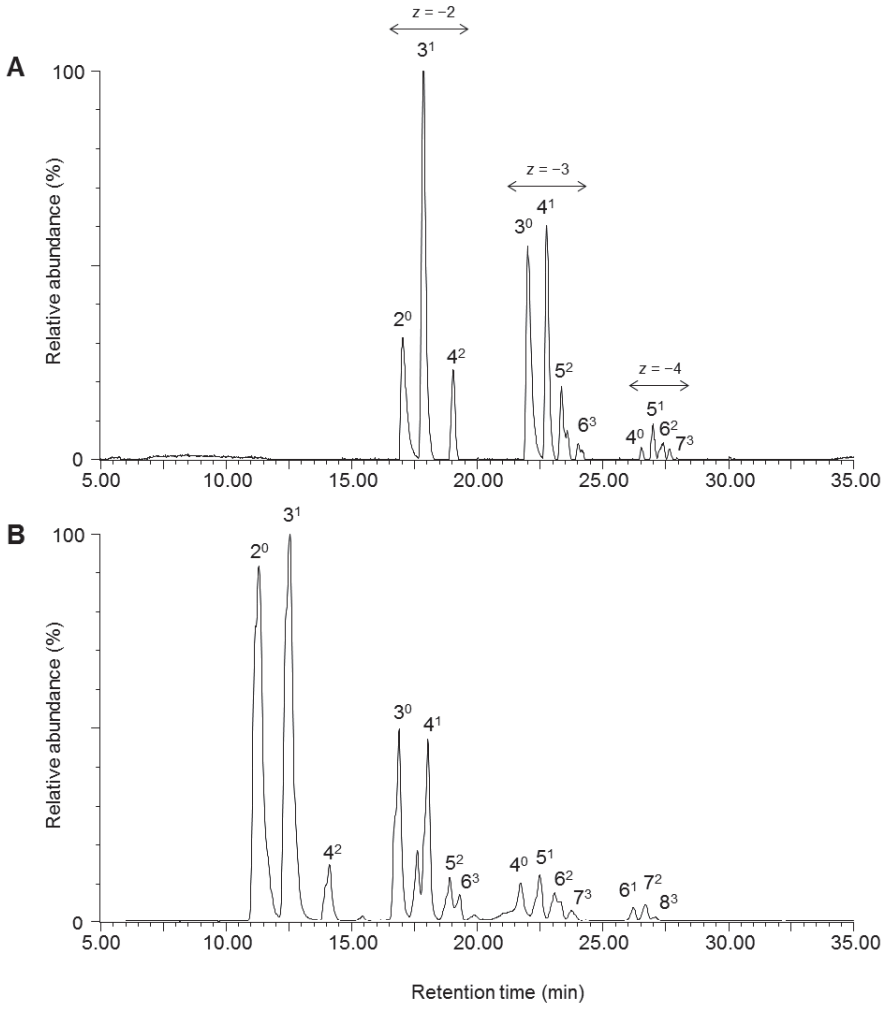

Figure 5.1. HILIC-MS base peak chromatograms of partially methylesterified α-(1,4)-linked galacturonic acid oligosaccharides derived from a DM30 pectin after treatment with endopolygalacturonase, (A) without labeling and (B) after fluorescent 3-AQ-labeling. Peak annotation: numbers, degree of polymerization; numbers in superscript, number of methyl esters. Total net charge (*z*) of the oligosaccharides is indicated in (A).

It was concluded that 3-AQ-labeling of pectic oligosaccharides improves the MS ionization efficiency of the complete oligosaccharide mixture without losing resolution using HILIC. An additional benefit of the use of HILIC prior to MS-analysis of 3-AQ labeled oligosaccharides is that time-consuming purification of labeled samples to remove the excess labeling reagent and salts is not required. These components elute quickly and can be directed to the waste to prevent pollution of the MS-source.

5.3.2 Profiling of complex oligosaccharide mixtures using fluorescence detection

Labeling of oligosaccharides with 3-AQ enables in-line fluorescence (FLR) detection in combination with TWIMS. To challenge the 3-AQ-HILIC-FLR-TWIMS method for its suitability to separate and characterize complex mixtures of isomeric pectic oligosaccharides, a mixture of higher complexity than the DM30 lemon pectin digest was labeled with 3-AQ and subsequently analyzed. This oligosaccharide mixture was derived from a DM56 lemon pectin after treatment with endopolygalacturonase and pectin lyase. Therefore, it contained both saturated and unsaturated partially methyl esterified α-(1,4)-linked galacturonic acid oligosaccharides. Figure 5.2 shows the HILIC-FLR-MS chromatograms. Peaks were annotated based on the detected masses and the predicted *[10]* HILIC elution pattern. Most of the compounds detected by MS (Figure 5.2A) were also detected using FLR detection (Figure 5.2B). However, there were some remarkable differences in relative peak intensity (e.g. for peaks at retention times 11.5, 18.2, 19.4, 22.4 and 23.3 min). This is mainly due to the fluorescence detection, which is on molar basis and thereby providing relatively low abundant peaks for oligosaccharides with higher degrees of polymerization. The ionization efficiency of oligosaccharides using ESI-MS can differ and non-carbohydrate compounds present in the sample may compete or interfere with ionization *[7]*. This makes oligosaccharide quantification using MS troublesome. The signals obtained using fluorescence detection are not affected by the (non-)carbohydrate moieties. Therefore, fluorescence detection enables quantification on molar basis of the levels of individual 3-AQ labeled oligosaccharides present in a mixture. Quantification using MS would also require specific standards for the large variety of oligosaccharide structures that may be present in a pectin digest, which are mostly not available in pure form.

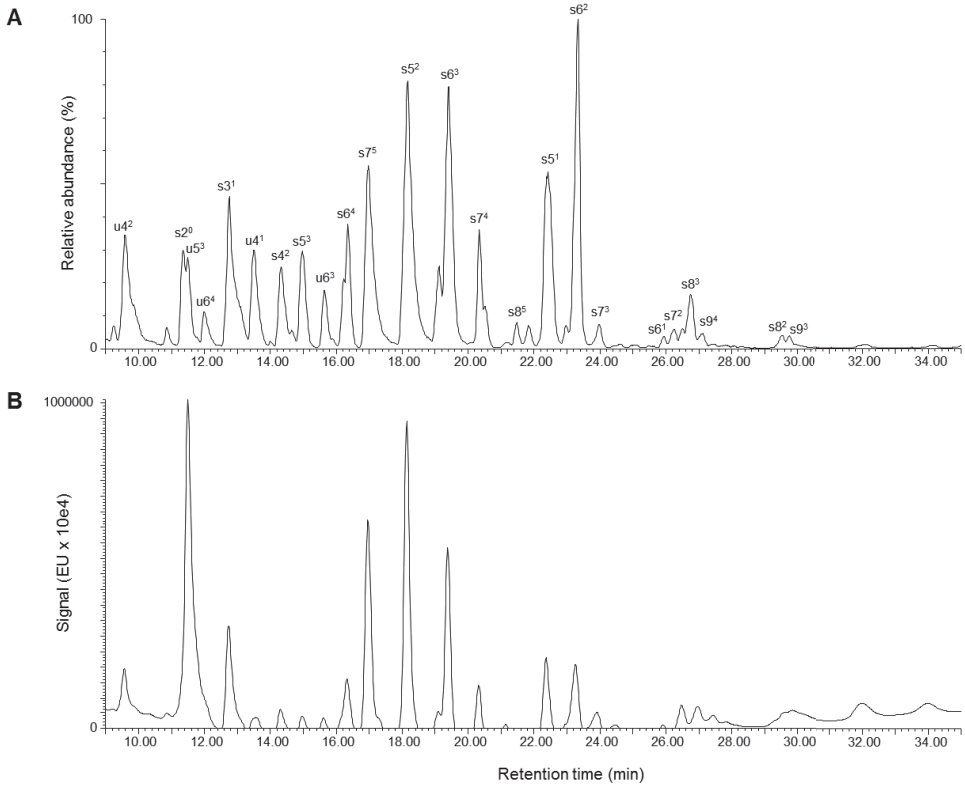

Figure 5.2. HILIC-FLR-MS chromatograms of 3-AQ-labeled saturated and unsaturated partially methylesterified α-(1,4)-linked galacturonic acid oligosaccharides derived from a DM56 pectin after treatment with endopolygalacturonase and pectin lyase. (A) Base peak chromatogram using MS and (B) chromatogram using fluorescence detection. Peak annotation: s, saturated; u, unsaturated; numbers, degree of polymerization; numbers in superscript, number of methyl esters.

5.3.3 Separation and characterization of isomers using 3-AQ-HILIC-TWIMS

Besides the HILIC-FLR and HILIC-MS chromatograms, IM data of the DM56 lemon pectin digest were collected during the same run. Using these IM data, it was possible to distinguish several isomeric structures. Figure 5.3 shows the drift time diagrams of several 3-AQ-labeled oligosaccharides. Each drift time diagram shows

a dominant IM peak, accompanied by 2-4 minor IM peaks. The different IM peaks are not fully separated, but the drift time diagrams clearly show the presence of several isomers for the 6^2, 7^2, 7^3 and 8^3 oligo-uronides. The drift time diagrams are different from diagrams as were reported previously for similar oligo-uronides *[16]*. This can be explained by the use of a different lemon pectin and enzymes with other specificities, which resulted in the formation of different isomeric oligosaccharides. The slight shoulder-shape of the dominant IM peaks indicated the presence of more than one isomer within the main peak. To confirm this, the product ion mass spectra obtained after different drift times using TWIMS in positive mode were evaluated.

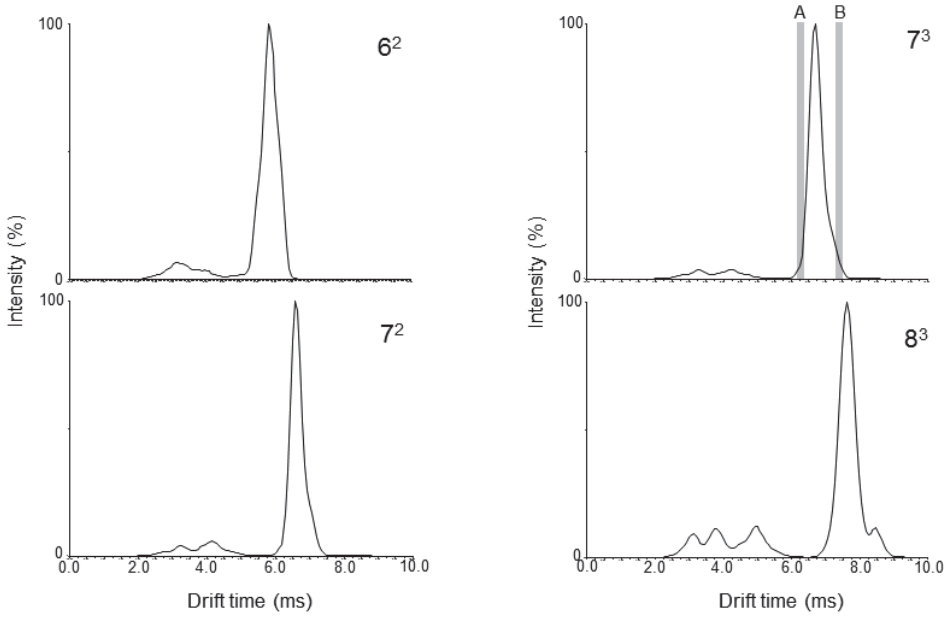

Figure 5.3. Drift time diagrams of different 3-AQ-labeled partially methylesterified α-(1,4)-linked galacturonic acid oligosaccharides, derived from an endopolygalacturonase/pectin lyase digest of a DM56-pectin: 6^2 (*m/z* 1229.3), 7^2 (*m/z* 1405.4), 7^3 (*m/z* 1419.4), and 8^3 (*m/z* 1595.4). Product ion mass spectra of the isomers with drift time windows A and B are shown in Figure 5.4.

Figure 5.4 shows the mobility selected product ion spectra of isomers within the dominant IM peak of the 7^3 oligosaccharide (as shown in Figure 5.3), using the drift time windows of 6.2-6.4 ms (Figure 5.4A) and 7.3-7.5 ms (Figure 5.4B). Interpretation was carried out as described previously *[16]*. In both Figures 5.4A

and 5.4B, one series of product ions with high intensity and at least one series with lower intensity were recognized, enabling the elucidation of the proposed structures of major and minor isomers of the 7^3 oligosaccharide. As described above, quantification of oligosaccharides using MS is difficult and it is unknown if variation in IM-response between isomers may occur. Therefore, we acknowledge that the terms 'major' and 'minor' do not necessarily reflect the actual quantitative ratios of the isomers present. Nevertheless, these terms are used for the sake of readability. Figure 5.4A shows a major isomer with methyl esterification on the second, fourth and fifth galacturonic acid residue, counted from the reducing end of the parental oligosaccharide. Additionally, a minor isomer with methyl esterification on the first, second and fourth galacturonic acid residue is shown. In Figure 5.4B, a major isomer with methyl esterification on the second, third and sixth galacturonic acid residue and a minor isomer, which carries a methyl ester on the third, fifth and sixth galacturonic acid residue, are shown. The presence of some product ions with a low intensity (e.g. *m/z* 673, 1039, 1229) indicates that even more isomers are present in both product ion mass spectra.

Within IM separations, the collision cross section (CCS or Ω) represents the effective area for the interaction between an individual ion and the neutral gas through which it is traveling *[12]*. CCS is an important characteristic of an ion in the gas phase, being related to its chemical structure and three-dimensional conformation. It affects the mobility of an ion as it moves through a neutral gas under the influence of an electric field. The theoretical CCS values (Ω_{th}) of the proposed structures of the 7^3 isomers in Figure 5.4 were estimated computationally and are given in Figure 5.4. The major isomer in Figure 5.4A shows a Ω_{th} of 292 $Å^2$, compared to a Ω_{th} of 304 $Å^2$ of the major isomer in Figure 5.4B. For adequate IM separation of isomers using the TWIMS instrument that was used in our study, isomers should differ in CCS by more than 5% according to the instrument specifications. As the difference in Ω_{th} of the isomers shown in Figures 5.4A and 5.4B is in some cases less than 5%, it explains why isomers were only partially separated and more than one isomer was present at the corresponding drift time window. Furthermore, it has been reported that theoretical CCS values can slightly differ from absolute CCS values obtained from calibrated drift time measurements *[14]*.

Based on the data obtained, it can be concluded that HILIC-TWIMS is very helpful for separation and characterization of a complex mixture of 3-AQ-labeled isomeric pectic oligosaccharides. Although the difference in CCS between some oligosaccharides was too small to separate all isomeric methyl esterified oligo-uronides completely, its separation capability was valuable for obtaining a proper indication of the isomers present and their corresponding structures. The theoretical CCS values can be of help to determine the separation potential of the

TWIMS instrument for different isomeric oligosaccharides. Nevertheless, the absolute CCS values as established during IM measurements may be slightly different from the theoretical CCS values.

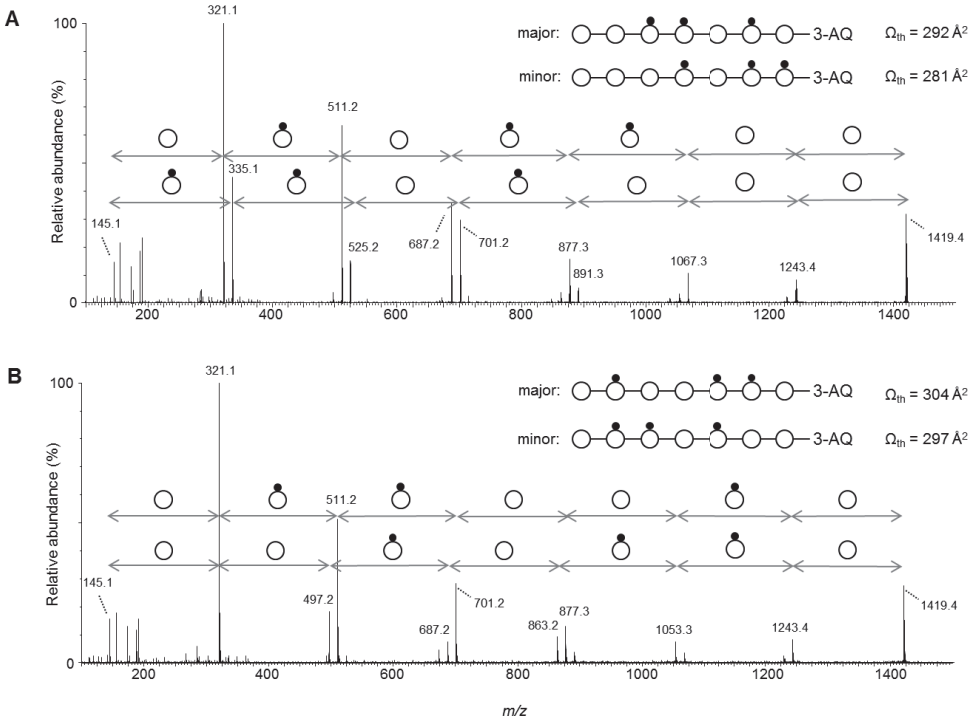

Figure 5.4. Product ion mass spectra of the 3-AQ-labeled 7^3 oligosaccharides (*m/z* 1419.4) derived from a endopolygalacturonase/pectin lyase digest of a DM56-pectin with drift time window of (A) 6.2-6.4 ms and (B) 7.3-7.5 ms, obtained using TWIMS in positive mode. Proposed structures and theoretical collision cross sections (Ω_{th}) are indicated. Legend: ○, galacturonic acid (176 Da); ●, galacturonic acid with methyl ester (190 Da); 3-AQ, 3-aminoquinoline.

5.3.4 Structure elucidation of recalcitrant isomeric sugar beet pectic oligosaccharides

As demonstrated above, the 3-AQ-HILIC-TWIMS method is valuable for simultaneous separation and characterization of mixtures of lemon pectin derived

oligosaccharides with anticipated isomeric structures. Other complex mixtures, such as digests of sugar beet pectin, can contain many unknown and unexpected structures. To further evaluate the strength of the 3-AQ-HILIC-TWIMS method, complex mixtures of recalcitrant isomeric sugar beet pectin derived oligosaccharides, obtained after enzymatic saccharification of sugar beet pulp, were analyzed using this method. Initially, the gross composition of this oligosaccharide mixture was determined, by analyzing its constituent monosaccharide composition. Table 5.1 shows that the oligosaccharides were mainly composed of galacturonic acid (42.6 % (w/w)), followed by rhamnose, galactose and arabinose. Glucuronic acid, glucose, mannose, xylose and fucose were present to a lesser extent. The proportion of glucuronic acid was relatively high, 4.9 % (w/w), since it has been estimated previously that only one out of 72 galacturonic acid residues within the rhamnogalacturonan backbone of sugar beet pectin is substituted with glucuronic acid *[24]*.

Table 5.1. Constituent monosaccharide composition of recalcitrant sugar beet pectin derived oligosaccharides.

Component	% (w/w)[a]
Galacturonic acid	42.6
Rhamnose	16.6
Galactose	16.5
Arabinose	12.4
Glucuronic acid	4.9
Glucose	4.4
Mannose	1.0
Xylose	0.7
Fucose	0.7

[a] dry matter basis

Figure 5.5 shows the base peak chromatogram of a mixture of 3-AQ-labeled sugar beet pectic oligosaccharides. The peaks are annotated with the proposed structures, based on the product ion mass spectra. The mixture mainly consists of rhamnogalacturono-oligosaccharides, partially acetylated and/or substituted with glucuronic acid. Several isomeric structures could be distinguished in the mixture using the IM data. The insert in Figure 5.5 shows, by way of illustration, the drift time diagram of the 3-AQ-labeled oligosaccharide with *m/z* 1181.3 that eluted at

19.2 minutes. Two IM peaks (A and B) with drift times of 6.2 ms and 6.8 ms could be distinguished, showing the presence of at least two isomeric structures for this oligosaccharide. To be able to elucidate the fine structure of these oligosaccharides, they were analyzed in negative MS detection mode. Fragmentation in negative mode provides more cross-ring cleavages compared to positive mode *[5]*. Thereby, it provides more diagnostic ions that are useful for revealing the precise location of the different substituents.

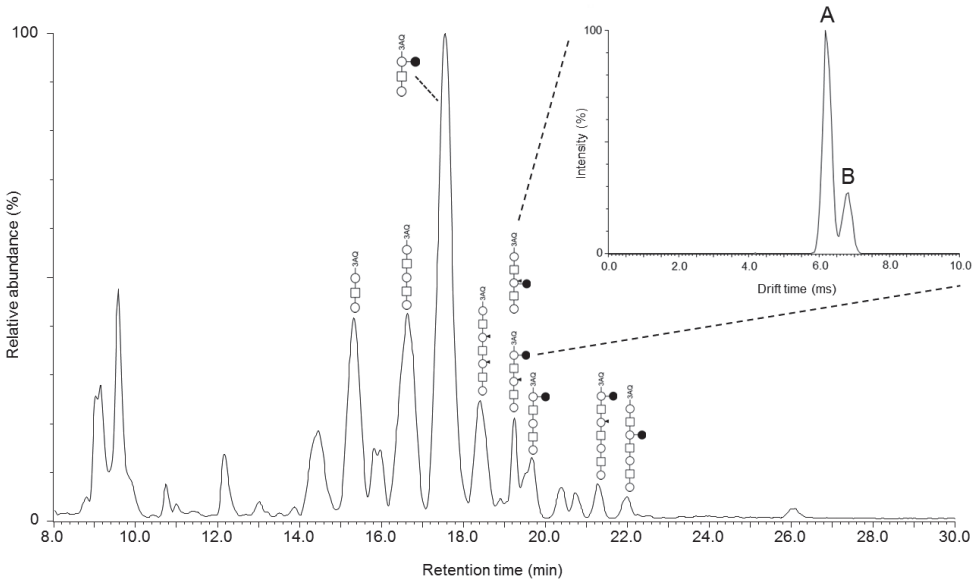

Figure 5.5. HILIC-TWIMS base peak chromatogram of a mixture of recalcitrant sugar beet pulp derived oligosaccharides. Insert: Drift time diagram of *m/z* 1181.3 with retention time of 19.2 minutes. Peak annotation: ○, galacturonic acid; ●, glucuronic acid; □, rhamnose; ▲, acetyl; 3-AQ, 3-aminoquinoline.

The product ion mass spectra of the *m/z* 1181.3 peak with drift time windows of 6.1-6.3 ms (IM peak A) and 6.7-6.9 ms (IM peak B) are shown in Figures 5.6A and 5.6B, respectively. They show the presence of the same ions, but with different intensities. This indicates that the same isomers were present in both IM peaks at different ratios. The major isomers that could be deduced from the obtained product ion mass spectra and their corresponding Ω_{th}-values are shown in Figure 5.6. The small differences in Ω_{th} of the isomers (311, 312 and 314 $Å^2$) explains why IM separation was incomplete. Based on the cleavages observed and the information in Table 5.1 concerning the presence of glucuronic acid, it was

deduced that the two major isomers (I and II) in Figure 5.6A were composed of a backbone of alternating galacturonic acid and rhamnose, with galacturonic acid present at both the reducing and non-reducing end of the oligosaccharide. The centre galacturonic acid residue carries an acetyl group and the galacturonic acid residue (on the former reducing end), labeled with 3AQ, is substituted with glucuronic acid. Based on the observed fragmentation (Figure 5.6B), another major isomer (III) could be sequenced with a different position of the glucuronic acid substituent compared to the structures I and II (Figure 5.6A). Both the glucuronic acid substituent and the acetyl group were attached to the centre galacturonic acid residue in the proposed structure in Figure 5.6B, which is a novel structural feature of sugar beet RG I.

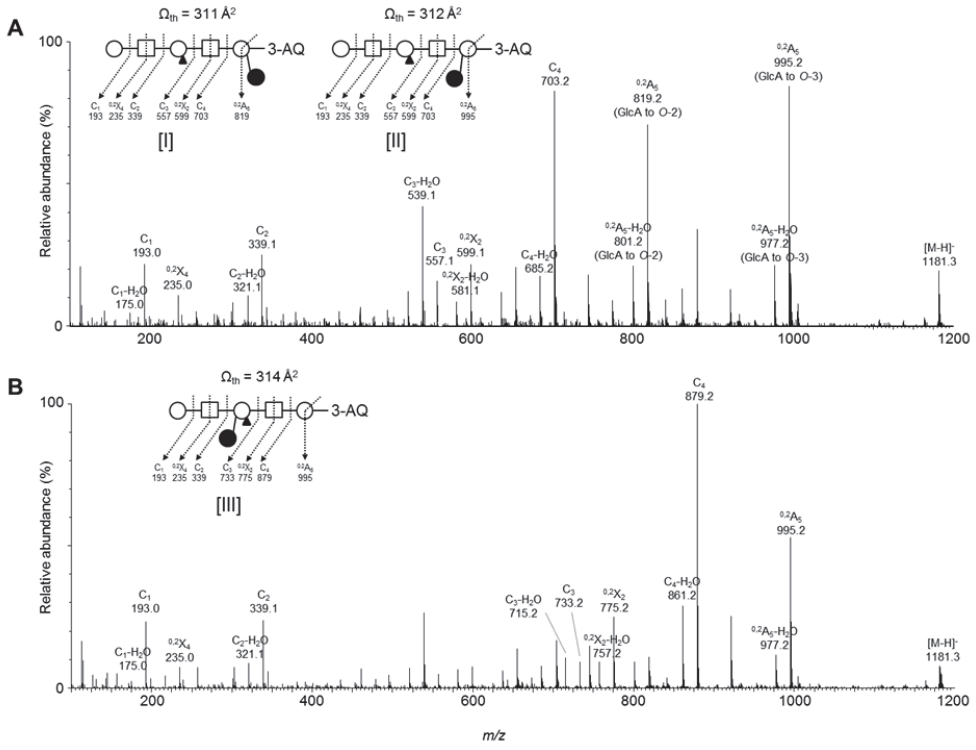

Figure 5.6. Product ion mass spectra of the 3-AQ-labeled sugar beet pectin derived oligosaccharides with *m/z* 1181.3 with drift time window of (A) 6.1-6.3 ms and (B) 6.7-6.9 ms, obtained using TWIMS in negative mode. Proposed structures (numbered I-III), observed cleavages and theoretical collision cross sections (Ω_{th}) are indicated. Legend: ○, galacturonic acid; ●, glucuronic acid; □, rhamnose; ▲, acetyl; 3-AQ, 3-aminoquinoline.

It has been described previously that ^{0,2}A- and ^{0,2}X-ions, derived from cross-ring fragmentation, can be obtained from RG I derived oligosaccharides using ESI-MS-analysis in negative mode. These ions are highly diagnostic for the linkage of the monosaccharide residues and the position of the different substituents *[5]*. In addition, water losses (−18 Da) can be observed frequently *[5]*. In Figure 5.6A, two different $^{0,2}A_5$-ions were clearly observed (995 and 819 Da). Based on these fragments, it is concluded that the glucuronic acid substituent can be attached to *O*-3 ($^{0,2}A_5$ = 995 Da) *or O*-2 ($^{0,2}A_5$ = 819 Da) of the galacturonic acid residue. So far, it has only been reported that glucuronic acid can be attached to *O*-3 of a galacturonic acid residue in sugar beet RG I *[24]*.
These findings show novel structural information about glucuronic acid substitution of sugar beet RG I, demonstrating that its structure is even more complex than was known already.

5.4 Conclusions

The combination of HILIC with TWIMS enables simultaneous separation and characterization of various complex mixtures of 3-AQ labeled isomeric pectic oligosaccharides. This newly developed method allows the precise identification of isomeric pectic oligosaccharides after separation by ion mobility and subsequent MS fragmentation. This method shows significant promise for the characterization of other complex samples containing isomeric oligosaccharides.

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Chapter 6

General discussion

6.1 Motivation of the research

This research was carried out as part of developing an industrial biorefinery process for sugar beet pulp. This biorefinery process comprises a targeted enzymatic hydrolysis of sugar beet pectin to release high amounts of its constituent monosaccharides, such as galacturonic acid and arabinose. They can be purified/isolated and subsequently transformed to multiple 'green' products. At the start of this PhD study it was already known that, apart from the monosaccharides released during enzymatic saccharification of sugar beet pulp, complex mixtures of oligosaccharides were obtained that had resisted enzymatic hydrolysis. Analysis of these complex mixtures of oligosaccharides appeared to be challenging and practically impossible without the use of time-consuming fractionation in combination with analytical techniques. Therefore, hydrophilic interaction chromatography (HILIC) coupled to evaporative light scattering detection (ELSD) and multidimensional mass spectrometry (MS^n) and HILIC coupled to traveling-wave ion mobility mass spectrometry (TWIMS) were developed as analytical methods. This enabled to monitor and the enzymatic saccharification of sugar beet pulp and to recognize lacking enzyme activities. To explore a possible outlet for the recalcitrant oligosaccharides that were obtained after enzymatic saccharification of sugar beet pulp, the *in vitro* fermentability of these types of oligosaccharides using human and pig fecal inocula was studied to investigate their potential to be utilized as functional food or feed ingredients.

6.2 Characterization of oligosaccharides using HILIC

6.2.1 HILIC-ELSD-MS^n

Enzymatic saccharification of biomass degrades the plant cell wall polysaccharides, resulting in hydrolysates consisting of complex mixtures of mono- and oligosaccharides *[1]*. As described in Chapter 1, several analytical techniques were already available at the start of this PhD study for the separation and characterization of such complex mixtures. However, all of these techniques have their own limitations for the simultaneous analysis of a broad range of plant cell wall derived oligosaccharides. Therefore, a HILIC-ELSD-MS^n method was developed as an additional technique for the analysis of a wide range of neutral and acidic plant cell wall derived oligosaccharides (Chapter 2). One of the main conclusions of this thesis is that HILIC-ELSD-MS^n currently is the most versatile and powerful analytical tool for oligosaccharide characterization.

HILIC separation capacity

The separation performance of HILIC for acidic plant cell wall derived oligosaccharides outperforms other techniques, as demonstrated in Chapter 2. Other techniques, like capillary electrophoresis, reversed phase and porous-graphitized carbon chromatography, fail to adequately separate acidic oligosaccharides having similar total negative charge, yet different degrees of polymerization, degrees of substitution or degrees of esterification. Using HILIC, we were able to adequately separate a wide range of neutral *and* acidic plant cell wall derived oligosaccharides, such as α-(1,5)-arabino-, α-(1,4)-gluco, β-(1,4)-gluco-, β-(1,4)-manno, β-(1,4)-xylo-, β-(1,4)-galacto-, α-(1,4)-galacturono-, xylogalacturono- and rhamnogalacturono-oligosaccharides.

It was demonstrated by the use of on-line MS that important structural characteristics of the oligosaccharides, such as acetyl and methyl ester groups, are retained during HILIC-analysis. Furthermore, it was shown that retention of pectin derived oligosaccharides in HILIC is decreased substantially by the presence of methyl ester groups, acetyl groups and an unsaturated galacturonic acid residue at the non-reducing end of the oligosaccharide. This typical HILIC retention behavior greatly simplifies interpretation of HILIC chromatograms. Also, it proved to be very useful for distinguishing acidic pectic oligosaccharides having the same degree of polymerization and molecular mass, while carrying different types or numbers of esters, as was shown in Chapters 2, 3 and 4.

HILIC coupling to MS^n

The efficient on-line coupling of HILIC to MS^n enables full annotation of the peaks obtained after HILIC separation of complex oligosaccharide mixtures. Combining the information about the typical HILIC retention behavior of substituted/esterified oligosaccharides with the MS^n-data enabled efficient sequencing of unknown oligosaccharides in complex mixtures. This was clearly shown in Chapters 2 and 3 for complex sugar beet pulp hydrolysates. HILIC-MS^n enabled the determination of the precise location of methyl esters, acetyl groups and sugar substituents within the sugar beet pulp derived oligosaccharides.

HILIC coupling to ELSD

ELSD was applied as second detector, next to MS, to evaluate if ELSD could be used routinely without MS for separation optimization and quantification. As illustrated in Chapter 2, the elution patterns and peak intensities obtained by ELSD and MS were rather comparable. Nevertheless, ELSD is considered to be more appropriate than MS for quantification. This is because the ELSD response is proportional to the concentration of the analytes, whereas MS response is affected by analyte ionization efficiency *[2]*. Therefore, the combined on-line coupling of

HILIC to ELSD and MS^n enables both the quantification and identification of oligosaccharides within complex mixtures.

Applications of HILIC-ELSD-MS^n

After its introduction, the HILIC-ELSD-MS^n method has been successfully used to analyze different complex oligosaccharide mixtures.

The HILIC-ELSD-MS^n method was applied to separate, identify and quantify partially methyl esterified and acetylated oligogalacturonides obtained after enzymatic hydrolysis of sugar beet pectin *[3]*. The ELSD response was similar for saturated and unsaturated galacturonic acid oligomers and no effect on the ELSD peak area was observed for the presence of esters. Figure 6.1 shows the separation and peak annotation of a complex sugar beet pectin digest using HILIC-ELSD-MS^n. This figure illustrates that oligosaccharides containing unsubstituted galacturonic acid units can be completely differentiated from the oligosaccharides containing methyl esterified and acetylated galacturonic acid units. The good alignment between MS- and ELSD-chromatograms allowed peak identification using MS, and peak quantification using ELSD. This application of HILIC-ELSD-MS^n has resulted in the elucidation of the substitution patterns of pectins having different functionalities and the development of new descriptive parameters enabling the detailed characterization of various pectins differing in functionality, while having the same gross composition *[4]*.

As shown in Chapter 4, HILIC-MS^n also enabled to monitor the metabolic fate of sugar beet pulp derived oligosaccharides during *in vitro* fermentation using human and pig fecal inocula. Using this technique, it was possible to determine the amount and type of oligosaccharides that were present at different time points during the *in vitro* fermentations. This enabled to plot the degradation and utilization rate of the different classes of oligosaccharides, which provided valuable information about the degradation of different classes of sugar beet pulp derived oligosaccharides by human and pig fecal microbiota.

HILIC-MS^n has also been successfully used to separate and identify individual alginate oligosaccharides in the feces from alginate-fed pigs *[5]*. Using HILIC-MS^n, saturated and unsaturated alginate oligosaccharides with DP 2-10 could be distinguished, which enabled the determination of variation in alginate degradation between individual pigs and the recognition of specific enzymatic degradation mechanisms of the microbiota in the large intestine of pigs.

The examples above show that HILIC-ELSD-MS^n is a powerful method for monitoring the fate of oligosaccharides during fermentation. This enables the determination of their degradation rate and the identification of intermediate products that may accumulate or end-products that are formed, both in *in vitro* and *in vivo* models.

Recently, other successful applications of HILIC-MS^n have also been reported, such as the use of HILIC-MS for the analysis of prebiotic galacto-oligosaccharides *[6]* and milk oligosaccharides *[7]*.

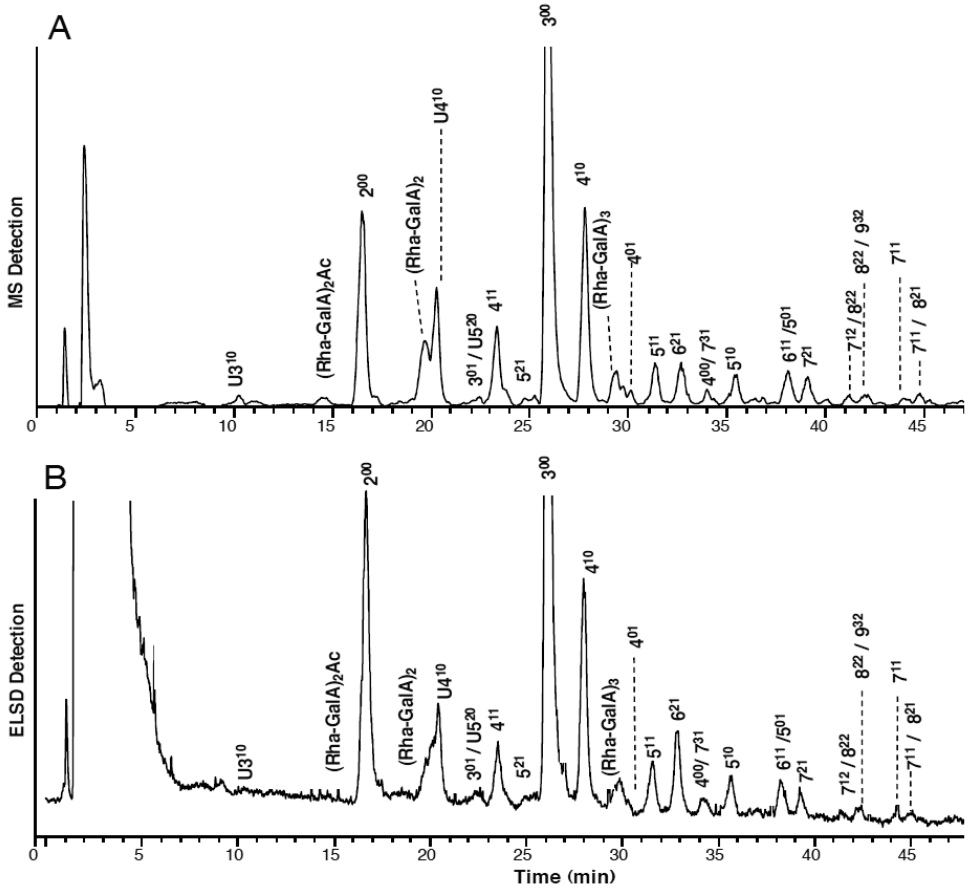

Figure 6.1. HILIC elution pattern of sugar beet pectin digested by rhamnogalacturonan I and homogalacturonan degrading enzymes using (A) MS^n and (B) ELS detection. Peak annotation: 5^{xy}, DP5-oligosaccharide carrying x methyl esters and y acetyl groups; U, unsaturated galacturonic acid at non-reducing end; Rha, rhamnose; GalA, galacturonic acid; Ac, acetyl group. Reprinted with permission from *[3]*.

6.2.2 HILIC-TWIMS

Although HILIC showed to be eminently suitable for the analysis of a wide range of oligosaccharides, it was observed during this research that its separation potential was insufficient to separate isomeric plant cell wall derived oligosaccharides within a complex mixture. Therefore, the coupling of HILIC to TWIMS is required to enable analysis of complex mixtures containing isomeric oligosaccharides.

As clearly demonstrated in Chapter 5, HILIC-TWIMS enables simultaneous separation and characterization of complex mixtures of various isomeric pectic oligosaccharides. This method allows the precise identification of isomeric structures after separation by ion mobility and subsequent MS fragmentation. It was shown that the complexity of the MS fragmentation spectra is reduced by labeling of the reducing end of the oligosaccharides with 3-aminoquinoline (3-AQ) prior to HILIC-TWIMS. Furthermore, 3-AQ labeling improved MS-ionization efficiency of the oligosaccharides without losing resolution of the HILIC-separation.

Using the newly developed 3-AQ-HILIC-TWIMS method, the methyl ester distribution within several isomeric lemon pectin derived oligogalacturonic acids was determined. Although the difference in collision cross section between the different isomers was in some cases too small to separate all isomeric methyl esterified oligogalacturonic acids completely, the TWIMS separation capability was valuable for obtaining a proper indication of the isomers present and their corresponding structures. It was shown in Chapter 5 that separation of isomers is required for unambiguous identification of isomeric oligosaccharides. For isomers having almost identical collision cross sections, this still poses a challenge.

The strength of the newly developed method was demonstrated with the analysis of complex mixtures of recalcitrant sugar beet pectin derived oligosaccharides (Chapter 5). Series of isomeric RG I derived oligosaccharides carrying a glucuronic acid substituent were characterized. This revealed novel structural information about the present linkages between glucuronic acid substituents and galacturonic acid in the backbone of sugar beet rhamnogalacturonan. Based on the MS-spectra obtained, it could be deduced that glucuronic acid was attached to *O*-3 or to *O*-2 of galacturonic acid units. Also, it was concluded that a single galacturonic acid unit within an oligomer may contain both an acetyl group and a glucuronic acid substituent.

The HILIC-TWIMS method shows an important promise for the identification of a wide range of isomeric oligosaccharides. For example, it can be applied for the analysis of other oligosaccharide mixtures that contain isomeric structures, for example human and animal milk oligosaccharides. This can contribute in understanding the structure-function relationships of these types of oligosaccharides *[8]*.

Quantification of 3-AQ labeled oligosaccharides using fluorescence detection
As described in Chapters 2 and 5, quantification of oligosaccharides using MS is troublesome due to differences in the ionization efficiency of oligosaccharides. Therefore, HILIC-TWIMS is appropriate for qualitative or semi-quantitative characterization of oligosaccharides. As demonstrated in Chapter 5, the labeling of oligosaccharides with 3-AQ enabled in-line fluorescence detection in combination with TWIMS. This enables quantification on molar basis of the levels of individual oligosaccharides present in a mixture, which is an additional advantage of 3-AQ labeling of oligosaccharides and an alternative to the quantification of the absolute amounts of oligosaccharides using ELSD as described previously *[3]*.

6.3 Enzymatic saccharification of sugar beet pulp

The research described in Chapter 3 aimed to optimize the enzymatic saccharification of sugar beet pulp on large scale to release the maximum amounts of galacturonic acid and arabinose with limited concomitant degradation of cellulose. An important conclusion is that 79% of the galacturonic acid and 82% of the arabinose can be released as monomers, while simultaneously degrading only 17% of the cellulose, using enzymatic saccharification conditions that are feasible for industrial upscaling.
Many previous studies on saccharification of sugar beet pulp (Table 6.1) had their limitations for efficient and cost-effective large-scale enzymatic saccharification. Some of the studies described incomplete degradation of the polysaccharides to monomers *[9-11]* or degradation of all the polysaccharides present *[9-17]*. The latter is undesired for production of galacturonic acid and arabinose with limited concomitant degradation of cellulose. Other limitations for cost-effective large-scale enzymatic saccharification observed in the studies were the use of high temperatures (≥120 °C) and/or the use of acids for pretreatment *[9, 14, 15, 17]*, the use of high enzyme dosages or the combined need of many different enzyme preparations *[12, 16, 18]*, the use of low (≤2.5% w/v) sugar beet pulp dosages *[9, 11, 19]* or the use of very long (≥72 h) incubation times *[10, 11, 15-19]*. Two of the studies presented in Table 6.1 *[18, 19]* describe a high yield of galacturonic acid and arabinose with limited cellulose degradation. However, the enzyme incubation time was long (96 h) and the solid to liquid ratios were very low (1-3.3% w/v). In our study we applied an incubation time of 48 h and a solid to liquid ratio of 7.5% w/v.
The overview presented in Table 6.1 clearly shows that the galacturonic acid and arabinose yields obtained in our study are higher compared to the yields presented previously with similar enzyme and sugar beet pulp dosages and relatively short (≤48 h) incubation times.

Table 6.1. Overview of studies on the saccharification of sugar beet pulp described previously. The limitations observed in the different studies for cost-effective large-scale saccharification of sugar beet pulp for the production of monomeric galacturonic acid and arabinose with limited concomitant degradation of cellulose are underlined.

Pretreatment	Enzymes / sources	SBP (% w/v)	Enzyme dosage/ g SBP	Time (h)	Efficiency / yield	Reference
none	**Viscozyme L, experimental preparation from *Aspergillus niger***	**7.5**	**10.66 µL (12.8 mg)**	**48**	**release of 79% galacturonic acid and 82% arabinose, 17% cellulose degradation**	***[20]* (this thesis)**
30 min, 121 °C, aq	*Trichoderma reesei*	2.5	89 FPU	48	90% hydrolysis total PS (not to monomers)	*[9]*
grinding	Maxazyme CL + Rapidase C80	9.8	20 mg + 25 mg	24	90% hydrolysis total PS	*[12]*
ball-milling and peracetic acid	*Talaromyces emersonii*	4	20.75 U	120	up to 77% hydrolysis total PS, 63% cellulose hydrolysis	*[15]*
pectinase (288 U/g SBP)	*Talaromyces emersonii, Trichoderma reesei*	4	25 FPU	120	92% hydrolysis total PS, 89% cellulose hydrolysis	*[10]*
grinding	SP 249 or Driselase	2.5	2 × 60 PGU	120	≥95% pectin hydrolysis (not to monomers), 60% cellulose hydrolysis	*[11]*
none	Pectinase SP 584	1	10 mg	96	release of 71% galacturonic acid and 67% arabinose, almost no cellulose degradation	*[19]*
drying and grinding, Pectinase SP 584	Celluclast 1.5L, Cellulase & 11 other commercial preparations	3.3	10 mg	96	≥95% pectin hydrolysis, up to 20% cellulose hydrolysis	*[18]*
grinding	Celluclast 1.5L, Viscozyme L & 5 other commercial preparations	6	±45 U	168	up to 80% hydrolysis total PS	*[16]*
none	Celluclast 1.5L FG, Novozym 431, Viscozyme L	5	±94 U	24	85% hydrolysis total PS	*[13]*
15 min, 140 °C, aq	*Chrysosporium lucknowense* C1	5	10 mg	48	>85% hydrolysis of total PS	*[14]*
30 min, 120 °C, 0.66 % (w/w) H_2SO_4	Celluclast 1.5L, Novozyme 188, Pectinex Ultra SPL	6	15 FPU, 15 CBU, 60 PGU	72	93% hydrolysis total PS, 63% total reducing sugar yield	*[17]*

Legend: SBP, sugar beet pulp; PS, polysaccharides; FPU, filter paper units (cellulase activity); CBU, cellobiase units; PGU, polygalacturonase units; U, enzyme units (µmol/min).

Efficient and cost-effective enzymatic degradation of polysaccharides is a crucial step in the biorefinery of biomass. Therefore, it is an important achievement of this research that high monosaccharide yields were obtained using enzymatic saccharification conditions that are feasible for industrial upscaling, i.e. no use of high temperatures and harsh chemicals, thereby preventing high energy consumption and excessive formation of salts. It is postulated that the high yield obtained without the use of pretreatments is due to the low lignin content of sugar beet pulp (1.8-3.4% w/w on dry matter basis, Chapter 1).
It was described in Chapter 3 that a further 5-10% yield increase could be expected if the enzyme incubation time was increased from 48 to 72 hours. Nevertheless, such a substantial increase of the hydrolysis time might reduce the economic feasibility for a large-scale process.

Recognition of lacking enzyme activities
MALDI-TOF-MS and HILIC-MS^n analysis enabled the characterization of the oligosaccharides that had resisted enzymatic hydrolysis. Based on these data and the release of the monosaccharides in time, important information could be deduced about enzyme activities present within the used pectinase preparations and, subsequently, lacking enzyme activities for hydrolysis of the recalcitrant oligosaccharides. It was described in Chapter 3 that especially rhamnogalacturonan galacturonohydrolase, arabinofuranosidase and pectin acetylesterase, able to remove acetyl groups from methyl esterified homogalacturonan, were lacking in the combined enzyme mixture.
During this PhD study, five commercial pectinase preparations, additional to the two preparations described in Chapter 3, were screened for their ability to degrade the recalcitrant sugar beet pulp derived oligosaccharides. These pectinase preparations were Rapidase ADEX D and Rapidase Liq+ (DSM Food Specialities, Delft, The Netherlands), Rhamnogalacturonase I and Vegazym P-CS (Erbslöh, Geisenheim, Germany) and Pectinex Ultra SP-L (Novozymes, Bagsvaerd, Denmark). These enzyme preparations are known to exhibit activity of various RG I degrading enzymes. Unfortunately, the observed additional release of monosaccharides was rather low (results not shown). This indicated that the lacking enzyme activities as described above were not generally present in commercial pectinase preparations or the action of the enzymes was hindered by the complexity of the recalcitrant oligosaccharide structures.
In Chapter 5, it was shown that the recalcitrant oligosaccharides, obtained after exhaustive enzymatic hydrolysis of sugar beet pulp, contained a relatively large proportion of glucuronic acid substituents. It was estimated that approximately one out of nine galacturonic acid residues was carrying a glucuronic acid substituent in the investigated hydrolysate. For sugar beet pectin, it has been reported that only

less than fifty galacturonic acid residues within the rhamnogalacturonan backbone carries a glucuronic acid substituent *[21]*. Therefore, it is hypothesized that the glucuronic acid substituents hinder the action of rhamnogalacturonan-degrading enzymes or rhamnogalacturonan acetylesterase, which causes recalcitrance of rhamnogalacturono-oligosaccharides substituted with glucuronic acid. Hence, it is recommended to search for enzymes that are capable of removing the glucuronic acid substituents from the backbone of sugar beet pulp rhamnogalacturonan. No enzymes are currently listed in the CAZy-database with this activity *[22]*. Nevertheless, the α-glucuronidases of family GH67 are able to remove glucuronic acid or 4-*O*-methyl-glucuronic acid from the non-reducing end of xylo-oligosaccharides *[23]*. It might be useful to investigate whether these enzymes exhibit activity on rhamnogalacturono-oligosaccharides substituted with glucuronic acid. Alternatively, the required enzyme activities for the degradation of recalcitant sugar beet pulp derived oligosaccharides may also be found by screening fermentation liquids from micro-organisms growing on these oligosaccharides as carbon source.

6.4 Utilization of sugar beet pulp derived oligosaccharides

As described in section 6.3, effort is needed to degrade the recalcitrant sugar beet pulp derived oligosaccharides in order to increase the monosaccharide yield. Alternatively, these oligosaccharides may be utilized as such as functional food or feed ingredients, since sugar beet pectin derived oligosaccharides (SBPOS) have been described as a promising prebiotic candidate. Different classes of SBPOS can be derived from sugar beet pulp, depending on the enzymes used to degrade the sugar beet pulp. Several studies have already indicated prebiotic functionality of specific classes of SBPOS, i.e. arabino-oligosaccharides *[24, 25]* and homogalacturono- and rhamnogalacturono-oligosaccharides *[26]*. The SBPOS that were obtained in our study mainly consisted of partially acetylated rhamnogalacturono-oligosaccharides and partially methyl esterified/acetylated homogalacturono-oligosaccharides. In Chapter 4, it was shown that these classes of SBPOS can be completely *in vitro* fermented by human and pig fecal microbiota, thereby producing short-chain fatty acids (SCFA). Mainly acetate and propionate were produced during *in vitro* fermentation, but also butyrate, which is believed to be beneficial for host health *[27]*. The degradation rate of SBPOS by pig fecal microbiota was lower compared to human fecal microbiota. In general, rhamnogalacturono-oligosaccharides were more slowly degraded than homogalacturono-oligosaccharides. The presence of acetyl groups within rhamnogalacturono-oligosaccharides lowered the degradation rate by pig fecal

microbiota, but not by human fecal microbiota. A classical prebiotic effect *[28]* could not be demonstrated for SBPOS, because no increase in bifidobacteria or lactobacilli was observed. Nevertheless, several other potentially beneficial modifications in the microbiota composition were observed during *in vitro* fermentation of SBPOS using human fecal inoculum. An increase of the growth of several bacteria that are associated with host health was observed, such as *Blautia* spp., *Coprococcus* spp., *Butyricicoccus* spp. and *Faecalibacterium* spp. Moreover, a decrease of the growth of several bacteria that may have adverse effects on host health and well-being was also noticed, such as *Sutterella* spp., *Alistipes* spp. and *Allisonella* spp. These are important findings, since it is increasingly recognized that besides the changes in bifidobacteria or lactobacilli, it is required to consider the changes of a much broader range of microbial groups with health promoting or disrupting activities to determine the prebiotic effect of specific oligosaccharides *[29]*.
Following the results presented in Chapter 4, it can be concluded that partially acetylated rhamnogalacturono-oligosaccharides and partially methyl esterified/ acetylated homogalacturono-oligosaccharides, obtained after enzymatic saccharification of sugar beet pulp, are promising candidates for application as prebiotic food or feed ingredient. Nevertheless, further *in vivo* studies would be needed to ultimately confirm the prebiotic properties of these types of oligosaccharides.

6.5 Biorefinery opportunities of sugar beet pulp

To minimize waste and maximize economic viability of the utilization of sugar beet pulp, a biorefinery approach should be followed, which is directed towards the valorization of *all* available components in sugar beet pulp. The results presented in this thesis show that it is feasible to produce different value-added products from sugar beet pulp. In Chapter 3, it was shown that monomeric galacturonic acid and arabinose and pectin derived oligosaccharides are obtained after enzymatic treatment. In addition, the insoluble fraction obtained after enzymatic treatment still contains most of the cellulose present in the pulp. Further research is needed to determine whether the mechanical properties of this remaining cellulose are still adequate for further application (e.g. as nanocomposite material or additive for paper products). The different components obtained after enzymatic saccharification of sugar beet pulp can be subsequently transformed into a number of biobased products, as summarized in Figure 6.2. Obviously, this also requires the additional upscaling for conversion of the different intermediate compounds into the final products, which forms a major challenge.

The biorefinery process could be made flexible by adapting the enzymatic saccharification process to produce products that generate the highest value. For example, by lowering the degree of saccharification more pectin derived oligosaccharides are produced.

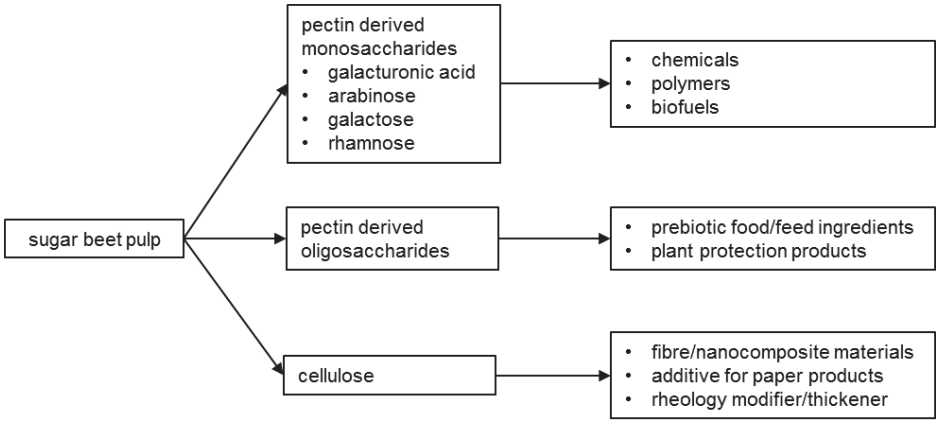

Figure 6.2. Biorefinery cascade of sugar beet pulp: simultaneous production of different value-added biobased products.

Over the last years, a number of changes have taken place within the European sugar sector, mostly driven by the reform of the European Sugar Regime *[30]*. It is expected that, due to this reform, sugar prices will become much more volatile in the near future. This increases the need for European sugar producers for extracting more value out of the sugar beet than is currently realized. To accomplish this, the valorization of sugar beet pulp is an important step forward. Inefficient and/or expensive enzymes and chemicals needed for degradation of plant cell wall polysaccharides and subsequent conversion of monosaccharides and oligosaccharides to several biobased products are current bottlenecks for viable biorefinery of sugar beet pulp. There is a large interest for biobased products, but prices should be competitive to traditional fossil fuel derived alternatives *[31]*. This forms a major challenge as long as fossil fuels are relatively cheap. Therefore, realizing the biorefinery opportunities for sugar beet pulp as described in this thesis will depend both on further optimization of the cost-effectiveness of the biorefinery process and on the future development of fossil fuel prices. The results obtained in this study can contribute to improving the economic feasibility of the biorefinery of sugar beet pulp by tailoring and optimizing the

enzymatic saccharification process to produce products that generate the highest value.

6.6 Implications of results for other fields of research

During this PhD research, a substantial effort was put into the development of novel LC-MS approaches, which can be used to characterize a wide range of both neutral and acidic plant cell wall derived oligosaccharides. As shown above, the use of HILIC-ELDS-MS^n was of great importance to be able to monitor the enzymatic saccharification of sugar beet pulp and to study the fate of SBPOS during *in vitro* fermentation. Furthermore, HILIC-TWIMS enabled to elucidate some novel structural features of sugar beet pectin. It is expected that other fields of research may also benefit from the results of this study. Some examples are discussed in this section.

Saccharification of biomass other than sugar beet
There are many different agro-industrial by-products, apart from sugar beet pulp, which are interesting feedstocks for biorefinery. The HILIC-ELSD-MS^n method used in this study to monitor the enzymatic saccharification of sugar beet pulp might also show its usefulness for facilitating the optimization of saccharification of other feedstocks, such as corn stover, wheat straw, sugar cane bagasse, potato fibre and chicory root pulp. Currently, recalcitrant oligosaccharides obtained after enzymatic saccharification of those feedstocks are mostly characterized using time-consuming fractionation of hydrolysates in combination with analysis of the fractions with different analytical techniques, such as HPAEC-PAD, MALDI-TOF-MS and NMR *[32, 33]*. In addition, HILIC-TWIMS might be useful for the identification of isomeric oligosaccharides.

Tailoring of oligosaccharides for specific applications
As described in this thesis, pectin derived oligosaccharides can be utilized for several applications. Tailoring of pectic oligosaccharides might be required for specific applications, because the chemical structure of pectic oligosaccharides may have a profound effect on their biological function *[34, 35]*. Tailoring can be performed by using pure and well-defined enzymes that target specific structures, e.g. deacetylation of homogalacturono-oligosaccharides *[36]*. HILIC-ELSD-MS^n can be used to characterize the oligosaccharides before and after enzymatic modification to study structure-function relationships.

Studying polysaccharide structure
Enzyme-assisted fingerprinting methods are powerful techniques to provide a rapid overview of the structure of various polysaccharides. The characterization of diagnostic oligosaccharides, which are formed by the degradation of polysaccharides by pure and well-defined enzymes, enables the elucidation of the parental polysaccharide structure. HILIC-ELSD-MS^n and HILIC-TWIMS are valuable additional techniques for analysis of complex mixtures of oligosaccharides derived from various polysaccharides. The usefulness of HILIC-ELSD-MS^n for characterization of such mixtures has been demonstrated recently for xanthan-derived *[37]*, alginate-derived *[5]* and chitosan-derived *[38]* oligosaccharides. In addition, HILIC-ELSD-MS^n has shown to be very useful for revealing the ester distribution patterns in acetylated pectins *[4]*. The additional use of HILIC-TWIMS can further improve the elucidation of the distribution patterns by the identification of isomeric oligosaccharides.

Enzyme screening and enzyme characterization
HILIC-ELSD-MS^n can be used as screening tool to identify the presence of enzyme activities in complex fermentation mixtures using recalcitrant or desired oligosaccharide structures. Besides this, fingerprinting of enzymatically modified polysaccharides is also a powerful tool for enzyme characterization. The mode of action of the enzymes can be deduced from the oligosaccharides profile that is obtained after incubation of the enzyme with its substrate. Recently, HILIC-ELSD-MS^n has been used to describe the mode of action of *Bacillus licheniformis* pectin methylesterase *[36]*. This was mainly achieved due to the unequaled HILIC separation potential for partially methyl esterified and acetylated pectic oligosacchairdes. In addition, HILIC-ELSD-MS^n was used to characterize a xanthan acetyl esterase from *Myceliophthora thermophila* C1 *[39]*. It is expected that HILIC-ELSD-MS^n and HILIC-TWIMS can be valuable tools for characterizing many different enzymes.

6.7 Future perspectives

In recent years, liquid chromatography hyphenated to mass spectrometry has become a key technique for oligosaccharide analysis *[40, 41]*. Future progress in oligosaccharide analysis will rely on continued development of both LC and MS techniques.
As shown in this thesis, HILIC coupled to MS is a versatile analytical tool for oligosaccharide characterization. Nowadays, different HILIC column materials are available, such as silica, diol or amide, each showing slightly different selectivities

towards various oligosaccharides *[42]*. So far, it has been difficult to adequately separate monosaccharides and oligosaccharides in a single run using HILIC, thereby hindering adequate quantification of reaction products and reaction kinetics. Furthermore, analysis of oligosaccharides with a degree of polymerization of more than nine is limited by the insolubility of these oligomers in the organic mobile phase solvents used in HILIC. Development of novel column materials may enable the use of mobile phases with less organic solvents. In addition, this may further increase the separation potential of HILIC or other types of chromatography. Next to this, the use of multidimensional LC can be of help to separate samples that current one-dimensional LC methods cannot adequately deal with *[43, 44]*. For example, the combination of RP×HILIC has been described before *[45]*. This type of 2D-LC might be useful for the analysis of complex mixtures of oligosaccharides which are partially feruloylated.
The use of different MS-techniques has been described in this thesis. It has been shown that ESI-IT-MS^n is a powerful tool for the characterization of oligosaccharides. The use of TWIMS enabled separation and identification of isomeric galacturono- and rhamnogalacturono-oligosaccharides. However, not all isomeric oligosaccharides could be separated, mainly due to very small differences in collision cross sections of the different isomers. Future developments in ion mobility MS may further increase the resolution of the separation of isomeric oligosaccharides. Nevertheless, it is expected that separation of isomers having almost identical collision cross sections will remain challenging.
Also, the interpretation of MS-spectra for the annotation of unknown structures forms a challenge in MS-analysis. Therefore, further development of specific software for the acquisition, processing and interpretation of mass spectra from plant derived oligosaccharides will be very useful.

In conclusion, the new analytical tools developed in this study open up new possibilities for the analysis of plant cell wall derived oligosaccharides in general. This can highly contribute to understanding the structure-function relationship of these oligosaccharides and elucidating the fine structure of the parental cell wall polysaccharides.

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Summary

Sugar beet pulp is a large volume by-product of the industrial beet sugar production. Currently, its main use is animal feed. Nevertheless, its composition provides interesting opportunities for increasing its economic value. This PhD-thesis was part of an industrial project that aims to develop a biorefinery process for the conversion of sugar beet pulp into multiple value-added 'green' products. Within this project, this thesis research focused on characterizing complex mixtures of sugar beet pulp derived oligosaccharides to be able to monitor and optimize the enzymatic saccharification of sugar beet pulp. In addition, the potential of sugar beet pulp derived oligosaccharides to be utilized as biofunctional food or feed ingredients was explored by studying their *in vitro* fermentability.

The background of the research is presented in **Chapter 1**. Information is provided on the composition of sugar beet pulp and its valorization opportunities. In addition, the saccharification of sugar beet pulp is discussed and enzymes needed for complete degradation of the sugar beet pulp polysaccharides are introduced. Furthermore, a review on the existing different analytical tools for the characterization of plant cell wall derived oligosaccharides is provided.

Analysis of complex mixtures of plant cell wall derived oligosaccharides is challenging and the use of time-consuming fractionation and multiple analytical techniques is often required for separation and characterization of these mixtures. Therefore, hydrophilic interaction chromatography with on-line evaporative light scattering detection and multidimensional mass spectrometry (HILIC-ELSD-MS^n) was developed as a versatile technique for the characterization of a wide range of neutral and acidic plant cell wall derived oligosaccharides (**Chapter 2**). Using HILIC, adequate separations were obtained for a wide range of neutral *and* acidic plant cell wall derived oligosaccharides, such as α-(1,5)-arabino-, α-(1,4)-gluco, β-(1,4)-gluco-, β-(1,4)-manno, β-(1,4)-xylo-, β-(1,4)-galacto-, α-(1,4)-galacturono-, xylogalacturono- and rhamnogalacturono-oligosaccharides. It was shown that the separation potential of HILIC for acidic oligosaccharides outperforms other techniques, such as capillary electrophoresis, reversed phase and porous-graphitized carbon chromatography. Important structural characteristics of the oligosaccharides, e.g. acetyl and methyl ester groups, are retained during HILIC-analysis. The retention of pectin derived oligosaccharides in HILIC is decreased substantially by the presence of methyl ester groups, acetyl groups and an unsaturated galacturonic acid residue at the non-reducing end of the oligosaccharide. This typical HILIC retention behavior makes interpretation of HILIC chromatograms remarkably easy. Also, it proved to be very useful for distinguishing acidic pectic oligosaccharides having the same degree of polymerization and molecular mass, while carrying different types or numbers of esters. Combining the obtained insights on oligosaccharide retention behavior in

HILIC with MS^n-data enabled efficient sequence elucidation of oligosaccharides in complex mixtures.

In **Chapter 3**, the enzymatic saccharification of sugar beet pulp for the targeted release of galacturonic acid and arabinose with minimal concomitant degradation of cellulose is described. By using a selected mixture of pectinases and enzymatic hydrolysis conditions that are feasible for industrial upscaling, it was possible to release 79% of the galacturonic acid and 82% of the arabinose as monomers from sugar beet pulp, while simultaneously degrading only 17% of the cellulose. This saccharification yield is much better compared to yields reported previously with similar enzyme and sugar beet pulp dosages, relatively short incubation times and without further heat or acid pretreatment. HILIC-ELSD-MS^n and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) were used to monitor the enzymatic saccharification of sugar beet pulp. By using these techniques, the recalcitrant oligosaccharides that were obtained after enzymatic saccharification were characterized. It was shown that the most abundant structures had an average degree of polymerization of 4-5. They were identified as partially acetylated rhamnogalacturono-oligosaccharides, mostly containing a terminal galacturonosyl residue on both reducing and non-reducing end, partially methyl esterified/acetylated homogalacturono-oligosaccharides, mostly containing methyl and acetyl esters at contiguous galacturonosyl residues and arabino-oligosaccharides, hypothesized to be mainly branched. It was concluded that especially rhamnogalacturonan-galacturonohydrolase, arabinofuranosidase and pectin acetylesterase are lacking enzymes for further degradation of these recalcitrant oligosaccharides.

To find a possible outlet for the recalcitrant sugar beet pulp derived oligosaccharides (SBPOS) that were obtained after enzymatic saccharification, the *in vitro* fermentability of these types of oligosaccharides using human and pig fecal inocula was studied to investigate their potential to be utilized as functional food or feed ingredient (**Chapter 4**). The metabolic fate of the different types of oligosaccharides and the changes in human fecal bacterial populations during *in vitro* fermentation were described. It was shown that SBPOS were completely fermented by human and pig fecal microbiota, thereby producing butyrate, next to the main metabolites acetate and propionate. The degradation of SBPOS by pig fecal microbiota was different and much slower compared to human fecal microbiota. In general, rhamnogalacturono-oligosaccharides were slower degraded compared to homogalacturono-oligosaccharides. Furthermore, it was observed that acetylation of rhamnogalacturono-oligosaccharides lowered the degradation rate by pig fecal microbiota, but not by human fecal microbiota. No classical bifidogenic effect was shown for SBPOS using human fecal inoculum. Nevertheless, several

other potentially interesting modifications in the microbiota composition that can be associated with host health are discussed.
In **Chapter 5**, the fluorescent labelling of oligosaccharides with 3-aminoquinoline (3-AQ) and the coupling of HILIC with traveling-wave ion mobility mass spectrometry (TWIMS) was introduced as an additional analytical technique for simultaneous separation and characterization of complex mixtures of various isomeric pectin derived oligosaccharides. This 3-AQ-HILIC-TWIMS method was used to determine the methyl ester distribution within several isomeric lemon pectin derived oligogalacturonic acids. Furthermore, a complex mixture of recalcitrant sugar beet pectin derived rhamnogalacturonan I (RG I) oligosaccharides was analyzed. Series of isomeric RG I derived oligosaccharides carrying a glucuronic acid substituent were characterized. Thereby, a number of novel structural features were identified for the first time: Glucuronic acid was attached to *O*-3 or to *O*-2 of galacturonic acid residues and a single galacturonic acid residue within an oligomer could contain both an acetyl group and a glucuronic acid substituent.
The main results that were obtained during this PhD research are discussed in **Chapter 6**. An overview of studies on the enzymatic saccharification of sugar beet pulp described previously, including their limitations for cost-effective upscaling is given. In addition, the latest insights on the biorefinery opportunities of sugar beet pulp and the relevance of this thesis for other fields of research are presented. Also, some future perspectives on the analysis of plant cell wall derived oligosaccharides are discussed.

Samenvatting

Suikerbietenpulp is een bijproduct van de bietsuikerindustrie en is in grote hoeveelheden beschikbaar. Het wordt momenteel voornamelijk als veevoeder gebruikt. De samenstelling van suikerbietenpulp biedt echter interessante mogelijkheden om de economische waarde ervan te verhogen. Dit promotieonderzoek maakte deel uit van een industrieel project wat als doel heeft om een bioraffinageproces voor de omzetting van suikerbietenpulp in meerdere waardevolle "groene" producten te ontwikkelen. Binnen dit project richtte dit promotieonderzoek zich vooral op de karakterisatie van complexe mengsels van uit suikerbietenpulp afkomstige oligosachariden, om het mogelijk te maken om de enzymatische sacharificatie van suikerbietenpulp te monitoren en te optimaliseren. Aanvullend werd de potentie van het gebruik van uit suikerbietenpulp afkomstige oligosachariden als biofunctionele (vee-)voedingsingrediënten onderzocht door het bestuderen van de *in vitro* fermenteerbaarheid.

In **Hoofdstuk 1** wordt de achtergrond van het onderzoek uiteengezet. Er wordt informatie gegeven over de samenstelling van suikerbietenpulp en de diverse mogelijkheden voor valorisatie. Daarnaast worden de sacharificatie van suikerbietenpulp en enzymen die nodig zijn voor complete afbraak van de polysachariden in suikerbietenpulp behandeld. Verder wordt een overzicht gegeven van de beschikbare analytische technieken voor de karakterisatie van oligosachariden die uit de plantencelwand afkomstig zijn.

Analyse van complexe mengsels van uit plantencelwanden afkomstige oligosachariden is een uitdaging en het gebruik van tijdrovende fractionering en de inzet van meerdere analytische technieken is vaak noodzakelijk voor scheiding en karakterisatie van deze mengsels. Daarom werd 'hydrophilic interaction' chromatografie met on-line 'evaporative light scattering' detectie en multidimensionele massa spectrometrie (HILIC-ELSD-MS^n) ontwikkeld als een veelzijdige techniek voor de karakterisatie van een brede reeks van neutrale en zure oligosachariden uit de plantencelwand (**Hoofdstuk 2**). Met het gebruik van HILIC werden effectieve scheidingen verkregen voor een uitgebreide reeks van neutrale *en* zure uit plantencelwanden afkomstige oligosachariden, zoals α-(1,5)-arabino, α-(1,4)-gluco, β-(1,4)-gluco, β-(1,4)-manno, β-(1,4)-xylo, β-(1,4)-galacto, α-(1,4)-galacturono, xylogalacturono en rhamnogalacturono oligosacchariden. Er werd aangetoond dat de potentie van HILIC voor het scheiden van zure oligosachariden die van andere technieken, zoals capillaire electroforese, 'reversed phase' en 'porous-graphitized carbon' chromatografie overtreft. Belangrijke structuurkenmerken, zoals bijvoorbeeld acetylgroepen en methylesters blijven intact tijdens HILIC-analyse. De retentie van uit pectine afkomstige oligosachariden in HILIC wordt substantieel verlaagd door de aanwezigheid van methylesters, acetylgroepen en een onverzadigde galacturonzuur eenheid aan het niet-reducerende uiteinde van het oligosacharide. Dit typische HILIC-retentiegedrag

vereenvoudigt de interpretatie van HILIC-chromatogrammen aanzienlijk. Hierdoor werd het mogelijk om zure pectine oligosacchariden die dezelfde polymerisatiegraad en molecuulmassa, maar een verschillend type of aantal estergroepen bevatten van elkaar te onderscheiden. De combinatie van het verkregen inzicht in het retentiegedrag van oligosacchariden in HILIC enerzijds en de verkregen MS^n-data anderzijds maakte een efficiënte opheldering van de oligosacharidenstructuren, zoals aanwezig in complexe mengsels, mogelijk.

In **Hoofdstuk 3** wordt de enzymatische sacharificatie van suikerbietenpulp voor het gericht vrijmaken van galacturonzuur en arabinose met een minimale gelijktijdige afbraak van cellulose beschreven. Door gebruik te maken van een geselecteerd mengsel van pectinases en enzymatische hydrolysecondities die geschikt zijn voor industriële opschaling was het mogelijk om 79% van het galacturonzuur en 82% van de arabinose uit suikerbietenpulp vrij te maken als monomeer, terwijl slechts 17% cellulose afgebroken werd. Deze opbrengst met vergelijkbare enzym- en suikerbietenpulpdoseringen, relatief korte incubatietijden en zonder verdere hitte of zuur voorbehandelingen is een stuk beter in vergelijking met eerder gerapporteerde opbrengsten. HILIC-ELSD-MS^n en 'matrix-assisted laser desorption/ionization-time-of-flight' massa spectrometrie (MALDI-TOF-MS) werden gebruikt om de enzymatische sacharificatie van suikerbietenpulp te monitoren. Met behulp van deze technieken werden recalcitrante oligosacchariden die verkregen werden na enzymatische sacharificatie van suikerbietenpulp gekarakteriseerd. Het werd aangetoond dat de meest voorkomende structuren een polymerisatiegraad hadden van 4-5. Zij werden geïdentificeerd als gedeeltelijk geacetyleerde rhamnogalacturono-oligosacchariden, voornamelijk met een eindstandige galacturonosyl eenheid aan zowel het reducerend als niet-reducerend uiteinde, gedeeltelijk methylveresterde en/of geacetyleerde galacturono-oligosacchariden, meestal methyl- en acetylesters bevattend aan opeenvolgende galacturonosyl eenheden en voornamelijk vertakte arabino-oligosacchariden. Het werd geconcludeerd dat met name rhamnogalacturonaan-galacturonohydrolase, arabinofuranosidase en pectine acetylesterase de ontbrekende enzymen zijn die mogelijk kunnen zorgen voor een verdere afbraak van deze recalcitrante oligosacchariden.

Om een mogelijk afzetkanaal te vinden voor de recalcitrante uit suikerbietenpulp afkomstige oligosacchariden (SBPOS) die verkregen waren na enzymatische sacharificatie, werd de *in vitro* fermenteerbaarheid van dit type oligosacchariden met gebruikmaking van humaan en varkens fecaal inoculum bestudeerd. Op deze manier werd de potentie van dit type oligosacchariden voor gebruik als functioneel (dier-)voedingsingrediënt onderzocht (**Hoofdstuk 4**). Het metabole lot van de verschillende typen oligosacchariden en de veranderingen in humane fecale bacteriepopulaties tijdens *in vitro* fermentatie werden beschreven. Het bleek dat

SBPOS volledig gefermenteerd werden door humane en varkens fecale microbiota, waarbij als metabolieten voornamelijk acetaat en propionaat, maar ook butyraat geproduceerd werden. De afbraak van SBPOS door varkens fecale microbiota verliep anders vergeleken met humane fecale microbiota. In het algemeen werden rhamnogalacturono-oligosachariden langzamer afgebroken in vergelijking met homogalacturono-oligosachariden. Daarnaast werd geconstateerd dat acetylering van rhamnogalacturono-oligosachariden de afbraaksnelheid door varkens fecale microbiota verlaagde, terwijl dit niet het geval was bij humane fecale microbiota. Er kon geen klassiek bifidogeen effect aangetoond worden voor SBPOS bij gebruik van humaan fecaal inoculum. Desalniettemin werden diverse andere potentieel interessante veranderingen in de microbiële samenstelling waargenomen die te relateren zijn aan gezondheid en welzijn van de gastheer.

In **Hoofdstuk 5** wordt de fluorescente labelling van oligosachariden met 3-aminoquinoline (3-AQ) en de koppeling van HILIC met 'traveling-wave ion mobility' massa spectrometrie (TWIMS) geïntroduceerd als een aanvullende analytische techniek voor de gelijktijdige scheiding en karakterisatie van complexe mengsels van diverse isomere uit pectine afkomstige oligosachariden. Deze 3-AQ-HILIC-TWIMS methode werd gebruikt om de methylesterverdeling binnen verschillende uit citroenpectine afkomstige isomere oligogalacturonzuren te bepalen. Daarnaast werd een complex mengsel recalcitrante uit suikerbietpectine afkomstige rhamnogalacturonaan I (RG I) oligosachariden geanalyseerd. Diverse series van uit RG I afkomstige isomere oligosachariden met een glucuronzuur substituent werden gekarakteriseerd. Daarbij werden enkele nieuwe structuren voor het eerst geïdentificeerd: Glucuronzuur was aan *O*-3 of aan *O*-2 van galacturonzuur eenheden gebonden en een enkele galacturonzuur eenheid binnen een oligomeer kon zowel een acetylgroep als een glucuronzuur substituent bevatten.

De belangrijkste resultaten verkregen in dit promotieonderzoek worden in **Hoofdstuk 6** bediscussieerd. Er wordt een overzicht gegeven van eerder gerapporteerd onderzoek naar de enzymatische sacharificatie van suikerbietenpulp, inclusief de daarbij aanwezige beperkingen voor kosteneffectieve opschaling. Aanvullend worden de laatste inzichten met betrekking tot de bioraffinagemogelijkheden voor suikerbietenpulp en de relevantie van dit proefschrift voor andere onderzoeksgebieden behandeld. Tot slot wordt een korte beschouwing gegeven op enkele mogelijke toekomstige ontwikkelingen op het gebied van analyse van oligosachariden, verkregen uit plantencelwanden.

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Martijn

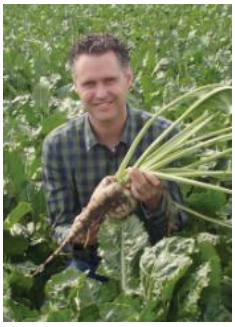
About the author

Curriculum vitae

List of publications

Overview of completed training activities

Curriculum vitae



Antonius Govardus Maria (Martijn) Leijdekkers was born on the 21st of February 1974 in Roosendaal. After graduating from secondary school (Atheneum B) at the Norbertus-college in Roosendaal in 1992, he started his study Food Technology at Wageningen University, specializing in Process Technology. After obtaining his MSc degree at Wageningen University in 1997, he worked for several companies within the food and beverage industry as quality and laboratory manager before he joined Cosun Food Technology Centre in Roosendaal in 2005 as manager of the analytical department. Since then, he was involved in different research activities within Cosun where he dealt mainly with carbohydrate analysis. In November 2009, he started his PhD study at the Laboratory of Food Chemistry of Wageningen University under the supervision of Prof. Dr Harry Gruppen and Prof. Dr Henk Schols. He performed his PhD study in part-time, while still working for three days a week for his employer. The results of his PhD research are presented in this thesis. Since September 2013, Martijn works as head of the analytical department at IRS, the Dutch institute for sugar beet research in Bergen op Zoom. Within this job, he specializes in projects that focus on chemical analysis, beet quality and storage.

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List of publications

Leijdekkers, A.G.M., Sanders, M.G., Schols, H.A., Gruppen, H. Characterizing plant cell wall derived oligosaccharides using hydrophilic interaction chromatography with mass spectrometry detection. *Journal of Chromatography A* **2011**, *1218*, 9227-9235.

Remoroza, C., Cord-Landwehr, S., **Leijdekkers, A.G.M.**, Moerschbacher, B., Schols, H.A., Gruppen, H. Combined HILIC-ELSD/ESI-MS^n enables the separation, identification and quantification of sugar beet pectin derived oligomers. *Carbohydrate Polymers* **2012**, *90*, 41-48.

Leijdekkers, A.G.M., Bink, J.P.M., Geutjes, S., Schols, H.A., Gruppen, H. Enzymatic saccharification of sugar beet pulp for the production of galacturonic acid and arabinose; a study on the impact of the formation of recalcitrant oligosaccharides. *Bioresource Technology* **2013**, *128*, 518-525.

Leijdekkers, A.G.M., Aguirre, M., Venema, K., Bosch, G., Gruppen, H., Schols, H.A. *In vitro* fermentability of sugar beet pulp derived oligosaccharides using human and pig fecal inocula. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 1079-1087.

Leijdekkers, A.G.M., Huang, J.-H., Bakx, E.J.; Gruppen, H., Schols, H.A. Identification of novel isomeric pectic oligosaccharides using hydrophilic interaction chromatography coupled to traveling-wave ion mobility mass spectrometry. *Carbohydrate Research* **2015**, *Accepted for publication*.

Overview of completed training activities

Discipline specific activities

Conferences and meetings

27th ICUMSA Session, Berlin, Germany, 2010
Analysis of complex carbohydrates, Rhenen, The Netherlands, 2011
Study trip oligosaccharide/fiber analysis[2], Minneapolis, MN, USA, 2011
28th ICUMSA Session, Cambridge, UK, 2012
2nd Starch round table, Wageningen, The Netherlands, 2012
XIIIth Cell wall meeting[1], Nantes, France, 2013
Technical event sugar beet growing sector, Tienen, Belgium, 2013
Symposium developments in carbohydrate analysis[2], Amsterdam, The Netherlands, 2013
Sugar beet information days[2], Emmeloord/Berkel-Enschot, The Netherlands, 2013
74th IIRB Congress, Dresden, Germany, 2014
29th ICUMSA Session, Ribeirão Preto, Brazil, 2014

Courses

Summer course glycosciences[1], Wageningen, The Netherlands, 2010
Advanced food analysis[1], Wageningen, The Netherlands, 2010
Food and biorefinery enzymology[1], Wageningen, The Netherlands, 2011

General courses

Communicate and win, Bosschenhoofd/Breda, The Netherlands, 2009
Information literacy/Endnote, Wageningen, The Netherlands, 2010
Project management, Bosschenhoofd/Breda, The Netherlands, 2011
Scientific writing, Wageningen, The Netherlands, 2012

Optional activities

Preparation PhD research proposal, Wageningen, The Netherlands, 2009
PhD study trip[1,2], Singapore and Malaysia, 2012
Food Chemistry seminars, Wageningen, The Netherlands, 2009-2014
Analytical meetings Royal Cosun/IRS, Roosendaal/Bergen op Zoom, The Netherlands, 2009-2014

[1]Poster presentation
[2]Oral presentation

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