Characterisation of cell wall polysaccharides in bilberries and black currants
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Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG (Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid)
Characterisation of cell wall polysaccharides in bilberries and black currants

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op vrijdag 2 maart 2007
des middags te vier uur in de Aula
Hilz, Hauke

Characterisation of Cell Wall Polysaccharides in Bilberries and Black Currants

Ph.D. thesis Wageningen University, The Netherlands, 2007
with summaries in Dutch and German

ISBN 90-8504-624-6
Abstract

Hilz, Hauke  Characterisation of cell wall polysaccharides in bilberries and black currants
Keywords  bilberries; Vaccinium myrtillus; black currants; Ribes nigrum; cell wall; polysaccharides; structure; rhamnogalacturonan II; xyloglucan; juice processing; high pressure processing

Cell wall polysaccharides play an important role during berry processing. Due to their polymeric character they can cause thickening after mashing of the berries and cause problems during extraction and clarification of fruit juices. Therefore, commercial enzyme preparations are used to degrade cell wall polysaccharides during processing. However, the berry cell wall composition of berries has never been studied in detail.

Aim of this thesis was to analyse cell wall composition and structure of bilberries and black currants to use the obtained data to monitor cell wall components during current processing and during high pressure processing.

A general characterisation of cell wall polysaccharides showed a higher pectin content with similar structure in black currants compared to bilberries. The major storage polysaccharides in black currant seeds were mannans and xylans in bilberry seeds. Rhamnogalacturonan II was enzymatically released in its dimeric form from all polymeric pectic populations of black currants and bilberries. Analysis of xyloglucans with different analytical techniques showed major differences between bilberries and black currants: while xyloglucan structure in black currants was simple, it was very complex in bilberries due to the presence of a rare side chain in partly novel building blocks.

During conventional processing using pectolytic enzymes, pectic polysaccharides were degraded to a large extent. Some polymeric pectic elements were extracted into the juice. One third of these polysaccharides were monomeric and dimeric rhamnogalacturonan II, two thirds were modified hairy regions. During high pressure processing pectins become more calcium sensitive and more extractable, which leads to the formation of a strong gel that hinders juice pressing. When high pressure is combined with commercial enzyme preparations, pectins are, however, further degraded than at atmospheric pressure.

The detailed characterisation of cell wall polysaccharides in bilberries and black currants led to the identification of novel structures and showed the complexity of the plant cell wall. Influences of novel processing technologies were now substantiated. A higher viscosity and lower juice yield were assigned to changes in pectin structure and extractability. This opens the possibility to use high pressure together with commercial enzyme mixtures to obtain a synergistic effect.
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Chapter 1

General Introduction
The project

Bilberries and black currants are popular fruits in northern Europe due to their attractive flavour and their strong colour. They are consumed as fresh berries or as diluted juice. Furthermore, juice (concentrate) and frozen berries are used as food ingredients. The research described in this thesis is carried out within the European project 'Novel enzyme-aided extraction technologies for maximised yield and functionality of bioactive components in consumer products and ingredients from by-products', acronym MAXFUN. The general objective of the MAXFUN-project was to develop novel processing technologies for the fruit and berry processing industry resulting in maximised exploitation of the quality and healthiness of the raw materials with concomitant improvement of the processability and minimisation of waste formation. A higher content of phenolic compounds in the juice is desirable, because phenolic compounds are colorants and are been conducted to be health promoting. Within this project the structure of the cell wall polysaccharides as well as the architecture of the cell wall were of interest, because the disruption of cell walls during berry homogenisation leads to the release of pectic polysaccharides, which form a highly viscous gel responsible for a low juice yield. To degrade this pectin gel, cell wall degrading enzymes are added to the berry mash. Treatment with cell wall degrading enzymes increases also the concentration of phenolic compounds in the juice. Improvement of the efficiency of these enzyme preparations needs detailed understanding of cell wall structure and architecture.

Bilberries and black currants

Bilberries (Vaccinium myrtillus L.) grow in coniferous forests and heathy districts of northern Europe and Asia (Franke, 1989; Grieve, 1994). They belong to the heath family (Ericaceae). Their botanical name originates from the Latin word baccinium for berry bush and from the similarity to the myrtle (myrtillus). The bushes are flowering in white from May until June and the blue, anthocyanin rich berries ripen from July till September. Bilberries are not cultivated but picked in the forest and contain red coloured pulp and juice (Baj et al., 1983; Franke, 1989; Grieve, 1994; Ebert, 2005).

Black currants (Ribes nigrum L.) originate from damp woods in northern Europe and have been cultivated since the 16th century (Grieve, 1994). They belong to the currant family (Grossulariaceae). Their Latin name originates from the Arabic word for currants (Ribes) and the black colour of the berries (nigrum). The bushes grow between one and two meters high and flower in April and May. The black berries are borne in clusters called 'strings' (Thompson, 1996) and ripen in July and August. The mechanically harvested berries are not consumed raw due to their unpleasant flavour, but processed to juice. Black currants contain high levels of flavonoids, mainly anthocyanins (Koeppen and Herrmann, 1977; Baj et al., 1983).
Chapter 1

Possible health effects

Hildegard von Bingen (1098 - 1179) was probably the first who described bilberries and their health effects: bilberries promote menstruation (BINGENESIS, 1533). Jacobus Theodorus (1520 - 1590) claimed that black currant juice strengthens gums and eases a swollen tongue, which was probably the first description of positive health effects of black currants (Tabernaemontanum, 1625). Today beneficial health effects of bilberries and black currants are attributed to their high anthocyanin contents. Anthocyanins are absorbed and excreted in urine as glycosides (Baj et al., 1983; Hou, 2003). Bilberries and bilberry extracts are claimed to strengthen connecting tissue and to have anti-inflammatory, anticancer, antimutagenicity, and apoptosis inducing activity (Morazzoni and Bombardelli, 1996; Hou, 2003). Black currants inhibit in vitro LDL oxidation, an early event of coronary heart disease (Heinonen et al., 1998b). It is reported that currants can prevent urinary tract infection due to their high flavonol content (Kontiokari et al., 2003) and due to a high molecular weight constituent, which prevents adhesion of Escherichia coli (Ofek et al., 1996). However, no evidence is presented that the constituent is absorbed by the intestine and excreted into the urine. Juice polysaccharides of black currants have been found to have possible immunostimulating and anti-tumour activity, as well (Takata et al., 2005).

Juice processing

A large part of bilberries and black currants is processed to juice, which can be consumed directly or used as a food ingredient. The Council of the European Union defined fruit juice as follows: The fermentable but unfermented product obtained from fruit which is sound and ripe, fresh or preserved by chilling, of one or more kinds mixed together, having the characteristic colour, flavour, and taste typical of the juice of the fruit from which it comes (2001/112/EC, 2002).

In the first step of juice processing berries are mashed. During mashing of berries, cell walls are disrupted and the released pectins form a highly viscous gel. To degrade this gel and improve the juice yield, industrial enzyme preparations, which are obtained from microorganisms, were developed and have been used in juice processing since the 1930s (Grassin and Fauquembergue, 1996). Continuous improvement of these enzyme preparations led to a wide variety of specific enzyme mixtures designed for the desired purposes. These developments changed juice manufacture from simple mechanical pressing or physical extraction to an accurate transformation technology with shortened production times and improved plant capacities (Grassin and Fauquembergue, 1996). In the European Union treatment with pectolytic, proteolytic, and amylolytic enzymes is permitted (2001/112/EC, 2002).

Commercial enzyme preparations contain a mixture of different pectolytic enzymes, which are classified according to their mode of action against pectic polysaccharides, which will be described in the following paragraphs. Polygalacturonases degrade non-methyl esterified homogalacturonan in an endo or
lyases degrade methyl esterified homogalacturonan in a trans eliminative way
(Albersheim et al., 1960), while pectate lyases degrade non-methyl esterified
polygalacturonan in the same way (Nagel and Vaughn, 1961b; Macmillan et al.,
1964). Rhamnogalacturonan hydrolase and rhamnogalacturonan lyase act on
rhamnogalacturonan I similar to polygalacturonase and pectin lyase on homog-
alacturonan, respectively (Schols et al., 1990a; Mutter et al., 1996). Pectin
methyl esterases release methanol from methyl esterified pectin. Fungal pectin
methyl esterases hydrolyse methyl esters most of the time randomly (Markovic
and Kohn, 1984; Benen et al., 2003), while endogenous plant pectin methyl
esterases hydrolyse methyl esters of homogalacturonan in a blockwise manner
(Schultz et al., 1945; Benen et al., 2003). Pectin acetyl esterases and
rhamnogalacturonan acetyl esterases remove acetyl groups from homo- and
rhamnogalacturonan I, respectively (Searle-Van Leeuwen et al., 1992; Searle-
Van Leeuwen et al., 1996).

By enzymatic degradation of berry cell walls not only the yield is improved,
but more flavonoids, which are beneficial for colour and probably for human
health, are released into the juice (Landbo and Meyer, 2001; Meyer, 2002;
Bagger-Jørgensen and Meyer, 2004). Especially anthocyanins located in the
vacuoles of epidermis cells become extractable after degradation of the cell walls
(Ros Barcelo et al., 1994). The polysaccharide composition within the cell walls
influences the extractability of these anthocyanins (Ortega-Regules et al., 2006).

For microbial stability juices are pasteurised after they have been packed.
Thermal treatment is the most common and easiest applicable method to
pasteurise or sterilise food. However, some sensorial parameters such as colour
and taste change after heating. Therefore, high pressure processing (HPP)
became of interest in the recent years. With pressures between 200 and 900 MPa
it is possible to increase the shelf life of a product with only minimal changes in
sensorial parameters (Hui, 2006).

The pure juice obtained from bilberries or black currants is too acidic to be
palatable. Therefore, bilberry juice is diluted to nectar with a final juice content
of 40 % and black currant juice to nectar with a final juice content of 25 %,
respectively, before consumption (2001/112/EC, 2002).

Within the MAXFUN project improvement of juice processing by the use of
pectolytic enzymes was the key target. For further improvement and monitoring
of changes, detailed structural composition of the cell wall is necessary. Therefore,
the literature describing the plant cell wall will be summarised in the
following paragraphs.
Figure 1.1: Schematic structures of plant cell wall polysaccharides.
General Introduction

The plant cell wall

The plant cell is surrounded by a cell wall, which is composed of the three different types of polysaccharides cellulose, hemicelluloses, and pectins (Voragen et al., 1995). Arabinogalactan proteins are also present next to small amounts of other structural proteins. The structural elements of the cell wall polysaccharides are shown in figure 1.1 and are described in the following paragraphs.

Cellulose

Cellulose is composed of β-1,4-linked glucose units and is the most abundant macromolecule in nature. The independent glucan chains aggregate and form microfibrils via hydrogen bonds. The native form of the glucan chains is cellulose I where the molecules are oriented parallel (Kroon-Batenburg and Kroon, 1997). These crystalline regions are diversified by amorphous regions. By mercerisation or regeneration cellulose is converted to the anti-parallel cellulose II form (Kroon-Batenburg and Kroon, 1997). Cellulose chains in cell walls of higher plants have a degree of polymerisation of 2000-6000 (Delmer, 1987).

Hemicelluloses

Xyloglucans are the major hemicelluloses of dicotyledons (Vierhuis et al., 2001; Hoffman et al., 2005) and are believed to cross link cellulose microfibrils (Keegstra et al., 1973; Hayashi et al., 1987). Three domains of xyloglucan are known: the first xyloglucan domain is present in free loops and cross links and is enzyme accessible, the second domain is hydrogen bond to the surface of the cellulose microfibrils and is extractable with concentrated alkali, and the third domain is entrapped in the amorphous cellulose microfibrils and is only accessible when cellulose is degraded (Pauly et al., 1999a). Xyloglucans have a regular structure of a β-1,4-linked glucan backbone. Three out of four glucose residues are substituted in position 6 with α-xyloses. Some of the xylose units can be further substituted with galactose, galactose-fucose, or arabinose (Bauer et al., 1973; Vierhuis et al., 2001).

Other hemicelluloses present in plants are glucuronoarabinoxylans and galacto(gluco)manans. Glucuronoarabinoxylans consist of β-1,4-linked xylan chains with arabinose and glucuronic acids attached (Brillouet et al., 1982). Galacto(gluco)mannans have a backbone of β-1,4-linked mannose units, which may be interrupted by glucose units, and galactose side chains attached to the mannose units of the backbone (Timell, 1964; Timell, 1965; Bremner and Wilkie, 1971).

Pectins

Pectins were first discovered in 1825 as a gelling acid and named after their properties with the Greek word for gelling acid: pectic acid (Braconnot, 1825a; Braconnot, 1825b). Today pectins are known to consist of a heterogeneous group of polysaccharides within the plant cell wall. They consist mainly of homogalacturonan and rhamnogalacturonan type I (RG I). Homogalacturonan is
a linear chain of α-1,4-linked galacturonic acid residues, whose carboxyl groups can be esterified with methanol and which can carry acetyl groups on O-2 and O-3 (Hirst and Jones, 1946; Worth, 1967; Pilnik and Voragen, 1970). First indications of the structure of RG I were given by linkage analysis of sycamore cells: galactan and arabinogalactan chains are attached to rhamnose units (Talmadge et al., 1973). RG I has a backbone of alternating 1,2-linked rhamnoses and 1,4-linked galacturonic acids. Side chains are attached to position 4 of the rhamnose (McNeil et al., 1980). The side chains, which are arabinan and arabinogalactan type I chains (Schols and Voragen, 2002), are very flexible molecules (Ha et al., 2005). Xylogalacturonans, which have a homogalacturonan backbone with single xylose units or longer 1,2-linked or 1,4-linked xylan chains attached to O-2 and O-3 of the galacturonic acid residues (Bouveng, 1963; Kikuchi et al., 1996; Le Goff et al., 2001; Nakamura et al., 2002), are probably also side chains of RG I (Schols et al., 1995a).

A special structural element of pectins is rhamnogalacturonan II (RG II). RG II consists of 12 different sugars among them the rare sugars 2-O-methyl xylose, 2-O-methyl fucose, aceric acid, 3-deoxy-D-manno-2-octulosonic acid (DHA), and 3-deoxy-D-lyxo-2-heptulosaric acid (KDO) (O’Neill et al., 1996). The sugar residues are interlinked by 20 different linkages (O’Neill et al., 1996). RG II has a well preserved structure, which hardly differs in structure in different plants (O’Neill et al., 2004) and is covalently linked to homogalacturonan (Ishii and Matsunaga, 2001).

**Arabinogalactan proteins**

Arabinogalactan proteins are rich in hydroxyproline and serin. To the latter arabinogalactan type II is attached (Lamport, 1970; Keegstra et al., 1973). These proteins are believed to be covalently linked to pectin (Immerzeel, 2005), but their physiological role is still unclear.

**Cross links**

Although individual cell wall polysaccharides have been characterised and their structures have been identified, the knowledge on cross links and interconnections of the different structural elements with each other and with other polysaccharides is limited. Pectins can be cross-linked via calcium bridges. The free carboxyl groups of homogalacturonan may form an ‘eggbox’ in which calcium is complexed as the ‘eggs’ (figure 1.2A) in dried and hydrogenated state (Grant et al., 1973; Morris et al., 1982). Another model describes the calcium-pectin complex anti-parallel pectin chains that form complexes with calcium (Walkinshaw and Arnott, 1981). These calcium cross-links can be the connection points of pectin gels or can hold pectin chains in the cell wall together (McCann and Roberts, 1991).

A second pectic cross link might be rhamnogalacturonan II (RG II). RG II can form dimers by esterification with boric acid as shown in figure 1.2B (Matoh et al., 1993; O’Neill et al., 1996; Mazeau and Perez, 1998; Ishii et al., 1999; Perez et
Figure 1.2 Polysaccharide cross links within the plant cell wall as described in literature. A) calcium-pectin-cross link as egg box model (Morris et al., 1982), B) rhamnogalacturonan II diester (Ishii et al., 1999), C) uronyl ester of pectin with an hydroxyl group of another polysaccharide chain (Lampert, 1970), D) 5-5-diferulic acid esterified with neutral side chains of pectin (Ralet et al., 2005), E) covalent linkage of xyloglucan to a galacturonic acid side chain of pectin (Keegstra et al., 1973).
al., 2003). Because RG II is covalently linked to homogalacturonan (Ishii and Matsunaga, 2001), a cross link of two pectin chains by RG II diesters is very probable. In various studies a covalent ester bond between uronic acid (galacturonic acid in pectins, glucuronic acid in glucuronoarabinoxylans) and hydroxyl groups of neighbouring polysaccharides was proposed as schematically shown in figure 1.2C (Lamport, 1970; Kim and Carpita, 1992; Brown and Fry, 1993; MacKinnon et al., 2002). In homogalacturonan more esterified galacturonic acids were determined than methyl groups (Kim and Carpita, 1992; McCann et al., 1994; MacKinnon et al., 2002). The non-methyl esters are probably formed between carboxyl groups of galacturonic acid and hydroxyl groups of sugars or sugar residues of polysaccharides. In spinach every 20th galacturonic acid residue is esterified to an unknown hydrophobic alcohol, which could act as a cross link of pectins (Brown and Fry, 1993).

Ferulic acid esters were first reported in xylans of wheat flour pentosans (Fausch et al., 1963) and are known to be present in monocotyledons ester linked to xyloglucans and arabinoxylans or in dicotyledons at the non-reducing ends of pectic arabinogalactan (figure 1.2D) (Geissmann and Neukom, 1973; Fry, 1983; Ishii, 1997). Diferulic acid bridges were reported to be attached to O-2 and O-5 of α-1,5-linked arabinan or O-2 of arabinose and O-6 of galactose from arabinogalactan (Ishii, 1997; Levigne et al., 2004; Ralet et al., 2005). These diferulic acid linkages can be inter or intra molecular. Recently ferulic acid dimers and trimers were identified, which are able to cross link two or three chains of arabinoxylans in monocotyledons (Allerdings et al., 2005; Bunzel et al., 2005a; Bunzel et al., 2005b; Funk et al., 2005).

Since the 1970s covalent cross links between pectins and xyloglucans have been discussed. Circumstantial evidence for a covalent cross link is co-elution of xyloglucans and pectins on size exclusion and anion exchange columns as well as cellulose binding of pectins in the presence of xyloglucans (Keegstra et al., 1973; Thompson and Fry, 2000; Popper and Fry, 2005). This covalent linkage was proposed to be formed via the reducing end of xyloglucan, because terminal galacturonic acid and not terminal glucose could be reduced in the complex (Keegstra et al., 1973). A possible structure is shown in figure 1.2E. However, neutral sugar side chains of pectin can non-covalently bind to cellulose, as well (Talbott and Ray, 1992; Zykwinska et al., 2005).

Proteins can also play a role in cross linking. Structural proteins become insoluble when secreted into the cell wall (Iiyama et al., 1994). Isodityrosine is a possible cross-link of extensin, but this may be only an intramolecular cross link (Fry, 1986).

Lignin might be esterified with carboxyl group of glucuronic acid or 4-O-methyl glucuronic acid in glucuronoarabinoxylans (Das et al., 1981) or might be bound via ferulic acid, which than can be oxidativly coupled to cinnamic acids of the lignin polymer (Iiyama et al., 1990).
Cell wall models

These different polysaccharide constituents and their possible cross links form the cell wall. The architecture, however, is not known. Over the years different models have been developed, which describe the plant cell wall and its architecture. The architecture has major influence on degradability and physical strength of the cell wall.

The first cell wall model described the cell wall of dicotyledons as one macromolecule, in which most of the different polysaccharides and glycoproteins (no direct evidence) are covalently linked (Keegstra et al., 1973; Albersheim, 1978). The only exception is the xyloglucan, which is attached to the cellulose microfibrils by hydrogen bonds. Because it is possible to extract part of the pectins from the cell wall with non-degrading solutions (Jarvis et al., 1981) and because different extraction procedures show that non-glycosidic bonds are involved in cohesion of cell wall polymers (Monro et al., 1976), this model cannot be completely right.

In onions, cell walls are described to have a cellulose-xyloglucan network and an independent pectin network (McCann and Roberts, 1991; Carpita and Gibeaut, 1993). At the same time, a slightly different model of the cell wall was described (Talbott and Ray, 1992). The difference is that the xyloglucan layer on cellulose microfibrils is covered by a second layer of arabinogalactans. Pectins are independent, but the chains fill the spaces between the cellulose microfibrils parallel to the microfibrils. These models were later modified, because NMR data suggested that the pectin network is not homogenous, but consists of ‘hard’ and ‘soft’ regions. These regions may fit between layers of cellulose microfibrils, so that the soft region is within and the hard regions are between the layers (Ha et al., 1997).

In monocotyledons the cell wall is of similar structure, although different polysaccharides are involved. Monocotyledons contain glucomannans in stead of xyloglucans and glucuronoarabinoxylans together with beta-glucans instead of pectins (Stone, 2006).

Aim of the research

The aim of the research described in this thesis was to characterise cell wall polysaccharides of bilberries and black currants. The cell wall composition and structure of neither bilberries nor black currants has been described in literature until now. A better understanding of pectin composition and structure within the cell walls of bilberries and black currants enables monitoring changes of cell wall polysaccharides during processing. This knowledge can be used to optimise juice production by physical or enzymatic treatments. Structure of the cell wall hemicelluloses in the pulp and skin is also of major interest due to their role in strengthening of the cell wall as part of the cellulose-xyloglucan framework.

Chapter 2 describes a general characterisation of polysaccharides present in the berries and in different tissues. A sequential extraction of the polysaccharides was performed to obtain fractions with pectins, hemicelluloses, and cellulose. The
pectic rhamnogalacturonan II (RG II) was further investigated in chapter 3, because RG II has a possible role as pectin cross link, which might be important for juice processing. Methods of xyloglucan analysis and the structure of xyloglucans from black currants and bilberries are described in chapter 4 and 5. These characterisations were carried out with the aim of getting a deeper insight in the diversity of xyloglucans and the biofunctional role of xyloglucan structure within the plant cell wall.

Another aspect of this thesis was the analysis of the effect of processing on cell wall polysaccharides. A characterisation of products from conventional processing is included in the general characterisation of berry polysaccharides (chapter 2). What happens to native RG II during conventional juice processing is described within the structural investigations of rhamnogalacturonan II (chapter 3). Juices and press cakes were analysed for RG II content and its mono or dimeric character. The effect of high hydrostatic pressure on cell wall polysaccharides alone and in combination with commercial enzyme preparations is described in chapter 6.

Chapter 7 summarises the results of the different chapters. In this chapter the implication of the results described in the previous chapters on berry processing and cell wall architecture are discussed, as well.
Chapter 2

Cell wall polysaccharides in black currants and bilberries - characterisation in berries, juice, and press cake

Published as
Cell wall polysaccharides in black currants and bilberries

Abstract

Cell wall polysaccharides from black currants and bilberries were characterised in three approaches. First, compositions of skin, pulp, and seeds show the distribution of polysaccharides over these tissues. A sequential extraction of cell wall material with different aqueous extractants informs about the extractability of the different polysaccharides, viz. pectins, hemicellulose, and cellulose. Finally, by isolation of cell wall polysaccharides from juice and press cakes obtained by the conventional juice manufacturing. The polysaccharide distribution was followed during juice processing. The main difference between bilberries and black currants is the dominant sugar residue in seeds: mannose for black currants and xylose for bilberries. Most of the hemicellulolytic sugars and cellulose can be found back in the press cake. The sugar composition of the press cake is similar to the composition of the residue after sequential extraction. Black currants contain more pectic sugars than bilberries. Consequently, a commercial enzyme used during processing releases more pectic material into the juice.

Keywords
vaccinium myrtillus; bilberry; ribes nigrum; black currant; cell wall polysaccharides; fractionation; juice processing; berries
Introduction

In the food industry most of black currants (Ribes nigrum L.) and bilberries (Vaccinium myrtillus L.) are processed to juice. This juice can be used directly for human consumption or as a food ingredient. With only pressing, juice yields from these berries are very low because a highly viscous pectin gel is formed after mashing. Therefore, cell wall degrading enzymes, mainly pectinolytic enzymes, are used during conventional berry processing (Grassin and Fauquembergue, 1996). An additional effect of enzyme treatment is improved colour extraction (Grassin and Fauquembergue, 1996), currently one of the most valuable characteristics of berry juice next to flavour.

In the future not only yield, flavour, and colour, but also the presence of biofunctional constituents might be of importance for berry juice. Anthocyanins and other polyphenolic compounds present in berries probably promote human health (Frankel, 1999). Next to polyphenols, cell wall polysaccharides may be valuable healthy compounds since they are belonging to the class of dietary fibre (Anderson, 1990; Yamada, 1994; Yamada, 1996; Sembries et al., 2003).

Plant cell walls consist of a firm network of hemicelluloses and cellulose, which is embedded in a matrix of pectins (McCann and Roberts, 1991). The main hemicellulolytic polysaccharides in dicotyledonous plants are xyloglucans, which mainly consist of a β-1,4-linked glucose backbone that is substituted at O-6 with xylose residues (McNeil et al., 1984). Pectic polysaccharides consist of two main structural elements: rhamnogalacturonan I (RG I, ‘hairy regions’) and homogalacturonan (HGA, ‘smooth regions’) (Voragen et al., 1995). Rhamnogalacturonan I contains a backbone of alternating rhamnose and galacturonic acid residues to which neutral side chains such as arabinans and type I and II arabinogalactans are attached (Schols and Voragen, 2002). Homogalacturonan is a long linear chain of 1,4-linked galacturonic acids, which can be methyl esterified or in position 2 or 3 acetylated (Quemener et al., 2003). Embedded in homogalacturonan is rhamnogalacturonan II (RG II) (Ishii and Matsunaga, 2001), which has a backbone of 8-10 galacturonic acids with four complex side chains consisting of 12 different sugars and 20 different linkages. Rhamnogalacturonan II can form a dimer through an apiose-boron ester (Kobayashi et al., 1996; Rodriguez Carvajal et al., 2003), which influences the pore size of the cell wall (Fleischer et al., 1999).

The first studies on cell wall carbohydrates in black currants and bilberries were limited to pectin contents, which vary between 0.20 and 1.79 g/100 g for black currants and between 0.10 and 0.78 g/100 g for bilberries (Lamptt and Hughes, 1928; Letzig, 1950; Money and Christian, 1950; Fuchs and Wretling, 1991). Salo and Suomi (1972) investigated hemicellulolytic sugars in bilberries and black currants and found high contents of mannose in black currant seeds and of xylose in bilberry seeds. However, they do not give a distribution of hemicellulolytic sugars over the different tissues of the berries.
During the juice manufacturing process high amounts of press cake are obtained. The press cake, which contains not only 70% of polyphenols originally present in berries (Meyer, 2002) but also large amounts of cell wall polysaccharides, might be a valuable source for health promoting and colour giving compounds. To date, press cake is burned, since its value is not accessible yet. However, some valuable compounds can be released using cell wall degrading enzymes (Meyer, 2002). On the one hand, this can lead to high quality juices with improved colour and potential health promoting effects and enables higher yields. On the other hand, the lower amount of press cake can be further upgraded by enzyme treatment to yield new products such as dietary fibre.

In this paper we present the first characterisation of cell wall polysaccharides in bilberries and black currants. We separated berries in skin, pulp, and seeds, extracted berry cell wall material with different aqueous extractants, and also included material from current juice processing in our study.

**Materials and Methods**

**Plant material**

Commercial berries, juice, and press cake from bilberries (Vaccinium myrtillus L.) and black currants (Ribes nigrum L.) were obtained from Kiantama Ltd., Finland. Damaged fruits were discarded. Frozen berries were separated into skin and pulp containing seeds. Pulp and seeds were separated after preparation of alcohol insoluble solids (AIS; vide infra). The press cake from black currants had to be separated into berry material and stams and wooden parts. The last part is further referred to as non-berry press cake (NBPC).

**Preparation of alcohol insoluble solids and sequential buffer extraction**

Cell wall material was precipitated with 70% aqueous ethanol (alcohol insoluble solids, AIS) and sequentially extracted with 0.05 M sodium acetate buffer at pH 5.2 and 70 °C (hot buffer soluble solids, HBSS), 0.05 M EDTA and 0.05 M sodium acetate in 0.05 M sodium oxalate at pH 5.2 and 70 °C (chelating agent soluble solids, ChSS), 0.05 M sodium hydroxide at 0 °C (diluted alkali soluble solids, DASS), and 6 M sodium hydroxide at 0 °C (concentrated alkali soluble solids, CASS) as described by De Vries et al. (1981) adapted by Vierhuis et al. (2000). The juice was ultrafiltrated through a 10 kDa membrane (Milipore Pellicon 2 MINI 10k B-10AQ 0.1 m²) in a Masterflex Consoledrive L/S 77250-62 system to obtain the polysaccharides from juice (juice MHR; vide infra).

**Sugar composition**

Sugar composition was determined after Saeman Hydrolysis. After a prehydrolysis step using 72% w/w sulphuric acid at 30 °C for 1h the samples were hydrolysed with 1 M sulphuric acid at 100 °C for 3 h. Afterwards the sugars were derivatised as alditol acetates and determined by gas chromatography (Englyst and Cummings, 1984) using inositol as internal standard.
Uronic acid content

The total uronic acid content was determined with the automated hydroxydiphenyl assay (Thibault, 1979). Differential determination of galacturonic acid and glucuronic acid was carried out by HPAEC after methanalysis according to De Ruiter et al. (1992).

Degree of acetylation and methyl esterification

Degree of acetylation and methyl esterification were determined by HPLC after hydrolysis with 0.4 N sodium hydroxide in isopropanol/water (50/50 v/v) (Voragen et al., 1986). The degree of acetylation and methyl esterification are calculated as mole methyl/acetyl groups per 100 mole galacturonic acid. One mole galacturonic acid can carry only one mol methyl esters and two moles acetyl ester.

Protein content

Protein content was determined using the combustion method (Dumas, 1831) on a Thermo Quest NA 2100 Nitrogen and Protein Analyser (Interscience, The Netherlands) according to the instructions of the manufacturer. 5 - 6 mg sample were weighed into a sample cup and directly analysed. D-Methionine was used as external standard. The protein content was calculated using 6.25 as nitrogen to protein conversion factor.

Soluble polyphenols

To determine the content of soluble polyphenols 1 ml water was added to 50 mg of the sample. This suspension was placed into an ultrasonic bath for 20 min and then centrifuged. The photometric absorption was measured after reaction with Folin-Ciocalteu-Reagent (Swain and Hillis, 1959).

Sugar Linkage analysis

Linkage analysis was carried out as described by Carpita and Shea (1989) adapted by Oosterveld et al. (1996). The samples were methylated and hydrolysed using 2 M TFA (2 h, 121 °C). After evaporation in a stream of air, the partially methylated sample was converted to alditol acetates and analysed by GC-FID. Identification of the compounds was performed using GC-MS.

High performance size exclusion chromatography (HPSEC)

High-performance size-exclusion chromatography (HPSEC) was performed on three Tosohaa TSK-Gel G columns in series (4000PWXL-3000PWXL-2500PWXL) using 0.2 M sodium nitrate as eluent (Chen et al., 2004).
Results and Discussion

Berries

The contents of dry matter and berry AIS, the latter representing cell wall material, show differences between black currants and bilberries. Both contents are higher for black currants when compared to bilberries (table 2.1). The determined values of dry weight material are in the range typically found for black currants (18.7 g/100 g) and bilberries (15.4 g/100 g) (Scherz et al., 2000).

Cell wall polysaccharides present in AIS can be characterised by their sugar composition (tables 2.2 and 2.3). The major sugar moiety in black currant AIS is galacturonic acid in contrast to bilberries, which contain glucose as major sugar. From the typical pectic sugars only galactose is present in both berries to the same relative amount. The relative amounts of rhamnose, arabinose, and galacturonic acid are larger in black currants. These findings indicate that less pectic material is present in bilberries (table 2.3). The galacturonic acid content found in bilberries is comparable to what has been found before for bilberries (Letzig, 1950; Fuchs and Wretling, 1991), low bush blueberries (Vaccinium angustifolium Ait.) (Chen and Camire, 1997), or blackthornton berries (Hippophae rhamnoides L.) (Dongowski, 1996). The galacturonic acid content found in black currants is comparable to the findings of Letzig (1950), but three to four times higher than what has been found by Fuchs and Wretling (1991).

Table 2.1 Relative amounts of the various tissues and processing products in fresh berries [g/100 g].

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<td>black currant non-berry press cake</td>
<td>6b</td>
<td>7.4c</td>
<td></td>
</tr>
</tbody>
</table>

|                          |       |       |      |
| bilberry skin            | 17.6  | 2.7   | 0.7  |
| bilberry pulp            |       | 1.4   |      |
| bilberry seeds           |       | 2.0   |      |
| bilberry pulp and seeds  | 60.0  | 7.9   | 3.1  |
| bilberry berries         | 100.0 | 12.4  | 3.8  |
| bilberry juice MHR       | 1.7   | 1.9   |      |
| bilberry press cake      | 3.2   | 37.0  |      |

a freeze dried after ultrafiltration
b % of fresh press cake
c AIS of non-berry press cake
Different berry tissues

Three different types of tissue comprise a berry: strong flexible skin, soft pulp, and firm and inflexible seeds. Since cell wall polysaccharides vary in different tissues within one fruit (Lecas and Brillouet, 1994; Vidal et al., 2001), these different tissues were separated (table 2.1).

Black currants were easily separated in pulp and seeds after drying of the total fruit. Due to smaller seeds this was not possible in bilberries. During separation of fresh tissue some berry material, mainly juice, was lost. The softer the pulp tissue the higher the loss: 22 g/100 g in bilberries compared to 5 g/100 g in black currant. The smallest part of berry AIS in bilberries and black currants originates from the skin, the largest from the seeds. Black currants contain more skin than bilberries and black currant skin contains more cell wall material (2.1 g/100 g skin) than bilberry skin (0.7 g/100 g skin) or grape skin (0.6 – 0.5 g/100 g skin) (Lecas and Brillouet, 1994). The weight content of seeds is higher and their size is larger in black currants than in bilberries.

Sugar composition

The sugar compositions of skin and pulp tissue differ only slightly in black currants, while in bilberries the pulp contains a larger amount of xylose residues when compared to the skin. The compositions of skin and pulp of both kinds of berries are similar to the findings for grapes (Lecas and Brillouet, 1994; Vidal et al., 2001).

In black currants a high mannose content distinguishes seeds from the other tissues, where mannose is almost absent. In contrast, seeds of bilberries contain mannose only in traces while xylose is the major sugar moiety present. Bilberry seeds contain double the relative amount of xylose when compared to pulp AIS. Salo and Suomi (1972) showed the same difference between black currants and bilberries. Bilberry seeds differ also form black currant seeds in the total amount of sugars, which is 70 % in bilberry seeds and 32 % in black currant seeds.

Degree of methyl esterification and acetylation

The degree of methyl esterification (DM) in pulp and skin (65 – 77 %) is slightly higher than in berry AIS (55 – 60 %) of both kinds of berries. The seeds of bilberries and black currants show a DM of approximately 40 %. These data correspond with a DM of 82.5 % found for pectins present in fresh black currant pulp and a DM of 57 % in mature black currants, respectively (Green, 1971).

In contrast, the degree of acetylation (DA) is much higher in bilberries than in black currants. With around 100 % the DA is extremely high in bilberry AIS. Although this is possible – per molecule uronic acid there are two binding sites for an acetyl group (O-2 and O-3) (Quemener et al., 2003) – in case of bilberries it is more likely that the acetyl groups are not only bound to galacturonic acid, but to hemicellulolytic polysaccharides, as well (vide infra).
Table 2.2 Sugar composition of black currants.

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*a* calculated as tannic acid, *b* total uronic acids, *c* EDTA may adulterates the result, *d* not determined, *e* values in brackets give mg/kg fresh berries.
Table 2.3 Sugar composition of bilberries.

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<th>total sugars [mol %]</th>
<th>Rha [mol %]</th>
<th>Fuc [mol %]</th>
<th>Ara [mol %]</th>
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a calculated as tannic acid, b total uronic acids, c EDTA may adulterates the result, d not determined, e values in brackets give mg/kg fresh berries.
Extracted fractions

Alcohol insoluble solids (AIS) of berries were sequentially extracted with different aqueous extractants. Seeds were left intact, as during juice processing. Four aqueous extractants were chosen: hot buffer releases not tightly bound pectins (hot buffer soluble solids, HBSS), while chelating agent solubilises pectins bound in cell walls via calcium (chelating agent soluble solids, ChSS). 50 mM sodium hydroxide releases pectins that are bound tightly to hemicellulloses or cellulose (diluted alkali soluble solids, DASS) and 6 M sodium hydroxide extracts hemicelluloses (concentrated alkali soluble solids, CASS). Cellulose and intact seeds remain in the residue. Tables 2.2 and 2.3 show how much material and total sugars were recovered in the five fractions. In both berries the residues are the largest fractions, which is partly due to the intact seeds. The amount of residue is larger in bilberries than in black currants, caused partly by a larger amount of seeds in AIS of bilberries (53%) when compared to black currant AIS (39%; table 2.1). The smallest fractions are CASS. Although bilberries gave higher AIS yields when compared to high bush blueberries (Vaccinium corymbosum L.), the yields of HBSS and ChSS from AIS are similar (Kader et al., 1994). However, the yield of DASS in high bush blueberries was less than half of the DASS yield of bilberries. This can be due to difference between the two species or to β-eliminative degradation of homogalacturonan at higher temperatures (Kravtchenko et al., 1992), as used by Kader et al. (1994).

Sugar composition

The sugar compositions of the extracted fractions inform about the extraction behaviour of different kinds of polysaccharides, viz. pectins and (hemi-)cellulose. HBSS and ChSS contain more than 80 mol% galacturonic acid (tables 2.2 and 2.3), which is the building block of homogalacturonan. Around 50% of galacturonic acid present in black currant and bilberry AIS is extracted in these two fractions. More or longer homogalacturonan segments are present compared to neutral segments ((Ara+Gal)/GalA > 0.12). Black currants contain more arabinose than galactose in HBSS and ChSS (Ara/Gal ≈ 3), while the relative amounts are equal in bilberries (Ara/Gal ≈ 1). Apples (Schols et al., 1995b) and olives (Huisman et al., 1996) contain more neutral segments compared to homogalacturonan segments, although the latter still predominate ((Ara+Gal)/GalA > 0.18 in apple and > 0.6 in olives). In the neutral segments arabinose dominates galactose (Ara/Gal ≈ 3 in apple and ≈ 7 in olives).

In DASS the relative amount of rhamnose is the largest of all fractions. The relative amounts of arabinose and galactose are large, either. This indicates the presence of large amounts of rhamnogalacturonan I, the pectic hairy regions. The relative amounts of galacturonic acid are large, although smaller than in HBSS and ChSS. DASS contain around 15% of all galacturonic acids present in berries. Pectins in black currant DASS contain more or longer neutral segments and less or shorter homogalacturonan segments than pectins in bilberry DASS ((Ara+Gal)/GalA = 0.62 and 0.29, respectively), but the Ara/Gal ratio is around 1.
and about the same in bilberry and black currant DASS. This was not the case in HBSS or ChSS.

With 6 M sodium hydroxide stronger hydrogen bounds are broken leading to the solubilisation of hemicelluloses (Roland et al., 1989). In black currant CASS xylose is the major sugar, followed by glucose and mannose. In contrast, bilberry CASS contain more glucose than xylose. The galacturonic acid content is the lowest of all fractions, so that the ratio of glucuronic acid to galacturonic acid is the highest in CASS. Glucuronic acid is commonly present in acidic hemicelluloses such as xylans (Rosell and Svensson, 1975). While in black currant CASS almost 50 % of the xylose is extracted, bilberry CASS contain only 6 % of the xylose present in berry AIS. Most of the xylose is present in the seeds, which remain in the residue. The same counts for the content of mannose in black currant CASS, which represents only 10 % of the mannose present in berry AIS.

In the residues the main sugar is glucose, but hemicellulolytic sugars play a major role due to their presence in seeds, as well. In black currant residue the content of mannose is even as high as the content of glucose and in bilberry residue the content of xylose reaches almost the content of glucose. Approximately 80 % of glucose and approximately 90 % of the major hemicellulolytic sugars - mannose in black currants and xylose in bilberries - present in berry AIS remain in the residue, which is an indication for limited extraction of cell wall polysaccharides from the seeds. Galacturonic acid is still present in the residues (11 mol%). In bilberries this amount represent still 35 % of galacturonic acid present in berry AIS, while this value is only 12 % for black currants.

Degree of methyl esterification and acetylation

For bilberries and black currants the DM is higher in HBSS than in ChSS, although ChSS of bilberries still have a DM of 77 %. This has been shown in olives and apple (Schols et al., 1995b; Huisman et al., 1996), as well, although chelating agents are supposed to extract calcium sensitive pectins, viz. pectin with a low DM (Ralet et al., 2003). Recent studies showed evidence that calcium is necessary to stabilise boron cross-linked rhamnogalacturonan II (dRGII-B) (Ishii et al., 1999; Kobayashi et al., 1999; Wimmer and Goldbach, 1999). Rhamnogalacturonan II is embedded in a homogalacturonan chain (Ishii and Matsunaga, 2001; Reuhs et al., 2004). The removal of calcium could destabilise dRGII-B, which could fall apart and set two pectic molecules free, independently of their DM. If this happens in muro, which still has to be proven, it can explain why ChSS contain high DM pectins.

Alkali treatment splits all methyl and acetyl ester linkages present. In HBSS and ChSS 82 % of the methyl groups and 28 % of the acetyl groups from black currant AIS are recovered. Thus, all methyl groups are linked to pectic galacturonic acids, the only sugar present that can form methyl esters. However, the missing acetyl groups can be either linked to galacturonic acids or to neutral
## Table 2.4 Sugar linkage composition of black currants.

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<th>DASS</th>
<th>CASS</th>
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*a* only 1,2,4-xylp
Table 2.5 Sugar linkage composition of bilberries.

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</table>

* only 1,2,4-xylp
polymers present in the (hemi-)cellulolytic fractions. In bilberries 84% of methyl groups, but only 3% of the acetyl groups are recovered in HBSS and ChSS. Too many acetyl groups remain in the other three fractions to be attached only to galacturonic acid. The main part of the acetyl groups has to be esterified with hemicellulolytic polysaccharides. This explains the high DA values for berry AIS and press cake AIS, because we calculated the DA as acetyl groups per galacturonic acid residues.

Sugar linkage analysis

To gain information about branching and structure of polysaccharides, the linkage composition was analysed (tables 2.4 and 2.5). Because some fractions are hardly soluble and almost all fractions contain large amounts of uronic acids, quantitative methylation was even after repeated methylation (three times) not possible. The results allow general conclusions about the polysaccharides present in the different fractions.

In all fractions less rhamnose was found than determined by alditol acetates (tables 2.2 and 2.3). This is due to a strong linkage between rhamnose and galacturonic acid in the rhamnogalacturonan I backbone, which cannot be hydrolysed with TFA. Most of the arabinose residues found in the different fractions are part of 1,5-linked arabinan side chains of rhamnogalacturonan I or type I arabinogalactan (Schols and Voragen, 2002). In the latter they can be attached to O-6 of the 1,4-linked galactan backbone. The contents of 1,4- and 1,4,6-linked galactose indicate that less type I arabinogalactans are present in HBSS and ChSS of black currants than of bilberries. The large amount of arabinose in black currants (tables 2.2 and 2.3) is probably due to arabinan side chains of pectins.

Bilberry HBSS and black currant HBSS and ChSS contain type II arabinogalactan, as concluded from a high content of 1,3,6-linked galactose. Hot buffer and chelating agent extract most of type II arabinogalactan next to type I arabinogalactan. With diluted alkali the remaining type I arabinogalactan is extracted. Type II arabinogalactans may not only be parts of pectic polysaccharides, but of arabinogalactan proteins, as well (Majewska-Sawka and Nothnagel, 2000). From black currant AIS more type II arabinogalactans are extracted with chelating agent than with hot buffer, while for bilberries it is the reverse.

The hemicellulolytic polysaccharides, viz. xylans, mannans, and glucans, are present as long linear 1,4-linked chains. Xylose, galactose, and fucose are present as terminal sugars, which is in combination with 1,4,6-linked glucose typical for xyloglucans (Vincken et al., 1994). Mannans, cellulose, xylans, and xyloglucans are present in black currants and bilberries.

Molecular weight distribution

High performance size exclusion chromatography (HPSEC) is an established tool to view the molecular weight distribution of soluble polysaccharides. In case
Figure 2.1 High performance size exclusion chromatography pattern of black currant polysaccharide fractions.

Figure 2.2 High performance size exclusion chromatography pattern of bilberry polysaccharide fractions.
of HBSS and ChSS in black currants and bilberries, which contain mainly acidic homogalacturonan, the first molecules elute in the excluding volume (figures 2.1 and 2.2). The chromatograms show a broad molecular weight distribution. With these non-destructive extraction conditions very large molecules, mainly homogalacturonans, are extracted. The molecular weight is comparable for HBSS and ChSS. The harsher the extraction conditions, the less polymeric are the polysaccharides in the corresponding fractions. Diluted alkali treatment opens bonds in pectic polysaccharides releasing them form hemicelluloses and leads to a shift in molecular weight. This shift can be due to extraction of natively smaller polymers or to slight degradation in diluted alkali. Concentrated sodium hydroxide extracts only some polysaccharides with very high molecular weight, while most of the extracted hemicellulolytic polysaccharides are of medium size. These trends are reported for olives (Vierhuis et al., 2000), as well. With the peaks at 31 min and 32 min in ChSS of black currants and DASS of both kinds of berries remaining EDTA is shown, which was not sufficiently removed during dialysis. The peaks do not represent cell wall polysaccharides.

**Material from juice processing**

The influence of current juice processing on cell wall carbohydrate composition and distribution is shown by a comparison of AIS from press cake with polysaccharides isolated from juice concentrate by ultra filtration (juice modified hairy regions - juice MHR: vide infra). Many different juices contain dissolved polysaccharides not degradable by enzymes used during processing (Schols and Voragen, 1994), although producers worked on the improvement of their enzymes to lower the amount of undegradable polysaccharides. These enzyme resistant pectic polysaccharides are called modified hairy regions. They contain rhamnogalacturonan I with arabinan and arabinogalactan side chains and stubs of homogalacturonan. Commercial enzyme mixtures used during juice production may have cut off some sugar residues.

In bilberries polysaccharide in press cake and juice account for 82 % of the total polysaccharides present in the berries. The losses are due to enzymatic degradation of homogalacturonans to small fragments which were lost during the ultra filtration step. The recovery is much lower in black currants (52 %) where more pectic polysaccharides are present. In black currants wooden parts and stems are present in the mechanical harvested bunches of black currants. They contribute up to 6 g/100 g of the press cake’s fresh weight and are further referred to as non-berry press cake (NBPC).

**Sugar composition**

The polysaccharides isolated from black currant and bilberry juice concentrate show the typical pattern of modified hairy regions (MHR) with large relative amounts of arabinose, galactose, and rhamnose (tables 2.2 and 2.3). Bilberry juice MHR contain less galacturonic acids than black currant juice MHR. The total sugar content is quite low in bilberry and black currant juice MHR (40 %), representing 6 % of total cell wall sugars present in black currant or bilberry AIS.
Juice MHR contain around 30% polyphenols measured by the colour assay, which can be more or less firmly attached to the polysaccharides or polymerised, giving juice MHR a red colour.

The compositions and contents of cell wall carbohydrates in the press cake are comparable to the composition of the residue after sequential extraction. From total cell wall sugars present in black currants 44% can be found in the residue and 46% in the press cake (70% and 76% for bilberries, respectively). Press cake AIS from black currants contain less mannose and more galacturonic acid when compared to the extraction residue.

With the enzyme treatment used it is possible to solubilise as many polysaccharides from AIS as could be extracted with the sequential extraction process. Up to 69% of the pectic sugars (galacturonic acid, arabinose, and galactose) are lost during juice processing due to enzymatic degradation. The power of the enzyme treatment is not only dependent on the kind of enzyme preparation used, but also on the kind of polysaccharide present in the fruits. In bilberries the composition of residue and press cake AIS are very similar, in black currants there are slight differences. The DM and DA of press cake AIS may indicate what can be expected for the esterification of polysaccharides remaining in the residue. For bilberry press cake AIS a DA of 157 seems very improbable confirming the assumption that acetyl groups are mainly bound to hemicellulolytic sugars.

However, it should be realised that non-berry material is present during processing of black currants in an industrial process. This non-berry press cake AIS (NBPC AIS) show a completely different pattern in sugar composition than all berry fractions. The tissue is much firmer and, as expected, NBPC contains a larger amount of glucose and xylose, viz. hemicellulose and cellulose, but less mannose when compared to berry AIS.

**Molecular weight distribution**

The juice MHR pattern is different for bilberries and black currants (figures 2.1 and 2.2). Both are similar till approx. 27 min, where an external standard of rhamnogalacturonan II elutes. The highest peak of black currant juice MHR elutes after approx. 29 min, while in bilberry juice MHR only a shoulder is detected. The HPSEC pattern of bilberry MHR is similar to the patterns of apple MHR (Schols et al., 1990b).

**Conclusions**

In this paper we are the first to present a detailed characterisation of cell wall polysaccharides in bilberries and black currants. Although the general tendencies are similar, some important differences were found.

The seeds of black currants contain mannose as major sugar, while bilberries contain xylose. After sequential buffer extraction mannose remains in the residue, where the whole seeds are recovered. The same counts for xylose in bilberries. Bilberries contain a much larger amount of residue than black
Cell wall polysaccharides in black currants and bilberries

currants, which can partly be due to the larger amount of seeds in bilberry AIS. Black currants contain more arabinose side chains in HBSS and ChSS.

The press cakes are in amount and composition very similar to the residues. With the enzyme preparation used it is possible to solubilise as many polysaccharides as could be extracted from berry AIS with the sequential extraction method. The polysaccharide fraction left in the juice show the typical composition of modified hairy regions as found in other fruits before.

Acetyl groups are present in a very large amount in bilberry polysaccharides that are extracted by alkali. These acetyl groups are mainly attached to hemicellulolytic sugars.

The findings described in this article are essential for the recognition of changes in cell wall polysaccharides during berry processing. Analysis of press cake AIS and juice MHR from conventional juice processing shows that the major part of cell wall polysaccharides remain in the press cake after juice processing (76 % in bilberries and 46 % in black currants, respectively).

**Acknowledgment**

This study has been carried out with financial support from the Commission of the European Communities, specific RTD programme 'Quality of Life and management of Living Resources', contract number QLK1-CT-2002-02364 'Novel enzyme-aided extraction technologies for maximized yield and functionality of bioactive components in consumer products and ingredients from by-products', acronym MAXFUN. It does not reflect its views and in no way anticipates the Commission's future policy in this area.

We thank Prof. Dr. Kaisa Poutanen and Dr. Marjaana Suutarinen, VTT Biotechnology, Finland, for the coordination of the MAXFUN project and Vernu Vasunta, Kiantama Ltd., Finland, for providing us with commercial berry material.
Chapter 3

The pectic polysaccharide rhamnogalacturonan II is present as a dimer in pectic populations of bilberries and black currants in muro and in juice

Published as
Abstract

Rhamnogalacturonan II (RG II) can play an important role during processing of berries due to its enzyme resistance and its possible role as a pectic cross-linker. This article describes the presence of RG II in cell walls, in juice, and in press cake of bilberries and black currants. RG II was identified and quantified via its diagnostic sugar residues. RG II, which was released from homogalacturonan, was probably present in its dimeric form in muro. Juice contained the free RG II dimer, while from press cake dimeric RG II was released by enzymatic degradation of homogalacturonan. A higher amount of RG II was present in juice than in press cake. During juice processing a cross-linker RG II might improve gel formation, which hinders the processability of berries. In addition, enzymes used during juice processing release dimeric RG II from pectin molecules and accumulate RG II in the juice.

Keywords
Vaccinium myrtillus; bilberry; Ribes nigrum; black currant; cell wall polysaccharides; rhamnogalacturonan II; juice; berries
**Introduction**

Bilberries and black currants are important crops in the Scandinavian countries. They have a strong and typical flavour and are rich in polyphenols (Buchert et al., 2005). Most berries are processed to juice. Due to the formation of a strong pectin gel after mashing, pectinolytic enzymes mixtures are used for juice (Grassin and Fauquembergue, 1996) and polyphenol extraction (Landbo and Meyer, 2001; Meyer, 2002; Bagger-Jorgensen and Meyer, 2004). After degradation with commercial pectinolytic enzymes, some polysaccharides resistant to enzymatic degradation remain in the juice (Hilz et al., 2005).

Pectic polysaccharides are a major part of the plant cell wall. They form a network independent from the hemicellulose/cellulose network (McCann and Roberts, 1991), and influence cell wall properties such as porosity or tensile strength (Fleischer et al., 1999; Titel and Ehwald, 1999; Ryden et al., 2003). Pectins consist of three major structural elements: homogalacturonan, which is a linear chain of α-1,4-linked galacturonic acids, rhamnogalacturonan I, which consists of a backbone of alternating α-1,4-linked galacturonic acid and β-1,2-linked rhamnose units (De Vries, 1988), and the complex rhamnogalacturonan II (RG II).

RG II is a well defined structural element of pectin. It is of low molecular weight and was first described in sycamore cells (Darvill et al., 1978). Its 12 different sugars that are connected via 20 different linkages make the polysaccharide the most complex known in nature to date. Attached to a backbone of 8 to 10 galacturonic acid residues (Melton et al., 1986; Vidal et al., 2000) rhamnogalacturonan II contains four side chains with rare and therefore diagnostic sugars: 2-O-methyl fucose (Barrett and Northcot, 1965; Darvill et al., 1978), 2-O-methyl xylose (Barrett and Northcot, 1965; Darvill et al., 1978), 3-C-carboxy-5-deoxy-L-xylose (acicric acid) (Spellman et al., 1983), 3-deoxy-D-lyxo-2-heptulosaric acid (KDO) (Stevenson et al., 1988), and 3-deoxy-D-manno-2-octulosonic acid (DHA) (York et al., 1985). In cell walls, the galacturonic acid backbone of RG II is unesterified when RG II is located near the plasma membrane and methyl esterified when RG II is located in the primary cell wall, as shown by immunolabelling (Williams et al., 1996). In the middle lamella no RG II could be immunolabelled (Matoh et al., 1998). The apiose units in side chain A (2-O-methyl xylose containing side chain) of two RG II monomers can be esterified with boric acid (Matoh et al., 1993; O'Neill et al., 1996; Mazeau and Perez, 1998; Ishii et al., 1999). This may be a covalent cross link between two pectin molecules in muro (O'Neill et al., 1996).

RG II can be released from homogalacturonan by enzymatic degradation with endo-polygalacturonase (Darvill et al., 1978; Ishii and Matsunaga, 2001). Polygalacturonase (PG) is involved in different processes in food manufacture such as vinification (yeast PG) or berry juice production (fungal PG as processing aid). During vinification RG II is released and not degraded by naturally occurring enzymes. This results in accumulation of RG II in wine, where it is
Rhamnogalacturonan II is present in bilberries and black currants stable for even more than 10 years (Doco and Brillouet, 1993; Doco et al., 1999). After liquefaction of fruits and vegetables, RG II is the dominant polysaccharide in apple, carrot, and tomato juice (Doco et al., 1997). Consequently RG II, if present in berry cell walls, is expected to be present in berry juice produced with the help of enzymes, as well.

The research described in this study is part of a project aiming at maximising yield and functionality of berry products by using novel enzyme-aided extraction technologies. Due to its presence in different juices, RG II has been a target of degradation studies with fungal enzyme extracts (Vidal et al., 1999). This study describes the presence of RG II in cell wall material of berries, juice, and press cake of bilberries and black currants. Pectins were sequentially extracted from cell wall material. In three obtained fractions the content of RG II was determined and a mass balance of RG II over the various pectic populations is presented. Finally, we investigated in which polysaccharide fractions RG II was bound to homogalacturonan and where it was present as monomer or as a dimer.

Materials and Methods

Material

Bilberries (Vaccinium myrtillus L.) and black currants (Ribes nigrum L.) and their commercial juice concentrates and press cakes were obtained from Kiantama Ltd., Suomussalmi, Finland. Alcohol insoluble solids (AIS), hot buffer soluble solids (HBSS), chelating agent soluble solids (ChSS), diluted alkali soluble solids (DASS), concentrated alkali soluble solids (CASS), the remaining residue and modified hairy regions (MHR) were prepared as described before (Hilz et al., 2005).

Neutral sugar composition as alditol acetates

Neutral sugars were determined as alditol acetates after TFA hydrolysis by GLC (Harris et al., 1984). Separation was carried out on a DB225 column (15 m x 0.53 mm ID; 1.0 µm film; J&W scientific) with helium as carrier gas (0.6 bar inlet pressure). Inositol was used as internal standard.

Sugar composition as trimethyl silyl derivatives

The determination of the sugar composition including common and rare acidic sugars, as present in RG II was done after methanolysis and derivatisation to trimethyl silyl (TMS) derivatives (Doco et al., 2001). The TMS derivatives were prepared using hexamethyldisilazane: trimethyl chlorosilane: pyridine, 3:1:9 (Syl-PREP Kit, Alltech), and analysed by GC-EI-MS using a Hewlett Packard mass selective detector 5970-B coupled to a Hewlett Packard 5890 GLC equipped with a DB-1 capillary column (30 m x 0.25 mm, 0.25 µm film; J&W Scientific). Quantification was done on a Carlo Erba HRGC 5160 system using the same column and flame ionisation detection.
Formation of monomers and dimers of RGII

RG II dimers were converted to the monomer in 0.1 M hydrochloric acid and back to the dimer by incubation in the presence of boric acid at pH 3.5 (Ishii et al., 1999). High-performance size-exclusion chromatography (HPSEC) was performed on three Tosohaas TSK-Gel G columns in series (4000PWXL, 3000PWXL, 2500PWXL) using 0.2 M sodium nitrate as eluent (Chen et al., 2004).

Pectin degradation with polygalacturonase in combination with pectin methyl esterase and with polygalacturonase in combination with pectin lyase

5 mg of the different fractions were dissolved in 1 mL sodium acetate buffer (50 mM, pH 5) and incubated with addition of 5 µL of endo-polygalacturonase (EC 3.2.1.15 from Kluyveromyces fragilis, 16 U/ml) and 1 µL of pectin methyl esterase (EC 3.1.1.11 from Aspergillus niger, 180 U/ml) or 5 µL of pectin lyase (EC 4.2.2.10 from Aspergillus niger, 8.8 U/ml) over night, respectively. The incubated samples were analysed on HPSEC and compared with non-treated material.

Results

Rhamnogalacturonan is present in all pectic fractions

Rhamnogalacturonan II (RG II) contains six diagnostic sugars, of which 2-O-methyl fucose and 2-O-methyl xylose were determined after hydrolysis with trifluoro acetic acid as alditol acetates using GC. Alcohol insoluble solids (AIS) and all three pectic fractions from bilberries and black currants contained 2-O-methyl fucose and 2-O-methyl xylose as minor sugar residues (tables 3.1 and 3.2).

Black currant AIS contained more 2-O-methyl fucose and 2-O-methyl xylose than bilberry AIS, probably because less pectin is present in bilberry AIS (Hilz et al., 2005). In bilberries and black currants, hot buffer soluble solids (HBSS) contained the highest amount of 2-O-methyl fucose and 2-O-methyl xylose of the three pectic fractions, whereas chelating agent soluble solids (ChSS) contained the smallest amount. 2-O-methyl fucose and 2-O-methyl xylose in the residue probably originated from pectic polysaccharides present in the seeds. The latter remained intact during extraction and were recovered in the residue. Although black currants contain higher amounts of seeds (Hilz et al., 2005), more 2-O-methyl fucose was found in bilberry residue than in black currant residue.

In the pectic fractions of bilberries 2-O-methyl xylose was present in similar concentrations as 2-O-methyl fucose. In most fractions of black currants, however, more 2-O-methyl fucose than 2-O-methyl xylose was present. This is in contrast to the commonly reported molar ratio of these sugars in an RG II molecule of 1:1. In the residue of bilberries and black currants even no 2-O-methyl xylose could be detected. The recovery of 2-O-methyl xylose in the different fractions is too high (147 %). Probably RG II was incompletely hydrolysed with TFA in AIS. With methanolysis followed by TMS derivatisation the obtained ratio was approximately 1:1, as expected (table 3.4). Therefore, the content of RG II was estimated on the basis of 2-O-methyl fucose. Although being
Rhamnogalacturonan II is present in bilberries and black currants

a minor component, RGII was present up to 9 % of polysaccharides in AIS and the different pectic fractions.

Table 3.1 Content of 2-O-methyl fucose, 2-O-methyl xylose, and rhamnogalacturonan II in pectic fractions of bilberries.

<table>
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<th>2-O-meth fucose</th>
<th>2-O-meth xylose</th>
<th>total CWPS</th>
<th>app. RG II</th>
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</thead>
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<tr>
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<td>[mg/kg berry]</td>
<td>[mg/kg berry]</td>
<td>[mg/kg berry]</td>
<td>[% of PS]</td>
</tr>
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<td>15238</td>
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<tr>
<td>HBSS</td>
<td>2 (29%)</td>
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<td>1464</td>
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</tr>
<tr>
<td>ChSS</td>
<td>1 (20%)</td>
<td>1 (13%)</td>
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<td>6</td>
</tr>
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<td>DASS</td>
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<td>1 (19%)</td>
<td>650</td>
<td>9</td>
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<td>0 (3%)</td>
<td>1 (17%)</td>
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<tr>
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<td>4 (67%)</td>
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</tr>
<tr>
<td>press cake AIS</td>
<td>3 (60%)</td>
<td>3 (50%)</td>
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</table>

in brackets sugars present in the fraction as % of sugar present in AIS

Table 3.2 Content of 2-O-methyl fucose, 2-O-methyl xylose, and rhamnogalacturonan II in pectic fractions of black currants.

<table>
<thead>
<tr>
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<th>2-O-meth xylose</th>
<th>total CWPS</th>
<th>app. RG II</th>
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<tr>
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<td>[mg/kg berry]</td>
<td>[mg/kg berry]</td>
<td>[mg/kg berry]</td>
<td>[% of PS]</td>
</tr>
<tr>
<td>AIS</td>
<td>19</td>
<td>9</td>
<td>34875</td>
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<tr>
<td>HBSS</td>
<td>9 (47%)</td>
<td>5 (48%)</td>
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<tr>
<td>ChSS</td>
<td>3 (15%)</td>
<td>2 (18%)</td>
<td>2463</td>
<td>7</td>
</tr>
<tr>
<td>DASS</td>
<td>5 (29%)</td>
<td>5 (57%)</td>
<td>3985</td>
<td>8</td>
</tr>
<tr>
<td>CASS</td>
<td>1 (4%)</td>
<td>0 (0%)</td>
<td>2344</td>
<td>3</td>
</tr>
<tr>
<td>residue</td>
<td>2 (12%)</td>
<td>0 (0%)</td>
<td>15179</td>
<td>1</td>
</tr>
<tr>
<td>juice MHR</td>
<td>12 (64%)</td>
<td>9 (95%)</td>
<td>2090</td>
<td>34</td>
</tr>
<tr>
<td>press cake AIS</td>
<td>9 (51%)</td>
<td>5 (51%)</td>
<td>15980</td>
<td>3</td>
</tr>
</tbody>
</table>

in brackets sugars present in the fraction as % of sugar present in AIS

By analysing juice modified hairy regions (MHR) and press cake (PC), it was possible to trace RG II during juice processing. In juice MHR of bilberries and black currants approximately the same amounts of the 2-O-methyl fucose and 2-O-methyl xylose were found. RG II is the major polysaccharide present, representing about 34 % of the total polysaccharides. In PC of bilberries 2-O-methyl fucose and 2-O-methyl xylose were present in the same amounts, while in black currant PC the amount of 2-O-methyl fucose exceeded the amount of 2-O-methyl xylose (similar to AIS). The content of these diagnostic sugars indicate that more RG II was extracted into the juice than remained in the press cake.

In AIS, HBSS, ChSS, DASS, the residue, juice MHR and PC AIS the diagnostic sugars (apiose, aceric acid, KDO, DHA) were unambiguously identified using GC-FID/MS after methanolation and derivatisation to TMS sugars. The cell wall fractions of bilberries (table 3.3) and black currants (table 3.4) contained the
diagnostic sugars in similar molar ratios, although in low amounts. With the exception of PC AIS of bilberries, 2-O-methyl fucose and 2-O-methyl xylose are present in the same amounts. The RG II molecule contains two apiose residues, one 2-O-methyl fucose residue, and one 2-O-methyl xylose residue, respectively. However, per mole 2-O-methyl fucose (2-O-methyl xylose, respectively) only one mole apiose was present. This is due to difficulties in peak separation of apiose and arabinose, which was present in 100 fold higher amounts. Difficulties in peak separation caused deviations in the molar percentages of the acidic sugars, as well.

**Rhamnogalacturonan II is present as a dimer in juice**

To investigate whether RG II is present as monomer or dimer in juice, RG II was monomerised and redimerised in bilberry and black currant juice MHR and analysed on by HPSEC. The dimer of RG II elutes in our HPSEC system after 27.0 min and the monomer after 27.8 min (figures 3.1 and 3.2).

When bilberry juice MHR was dissolved at pH 3.5, peaks at retention times of both monomer and dimer were present. After treatment of bilberry juice MHR with 0.1 N hydrochloric acid, the peak at 27.0 min disappeared, although the RI response did not reach the baseline. The borate-diol ester of the RG II dimer was hydrolysed by hydrochloric acid treatment. Apparently, other material with the same hydrodynamic volume was present, since pure RG II was converted quantitatively by the same treatment. The shoulder at 27.0 min seemed to be enlarged after acid treatment, due to the formation of monomeric RG II. After incubation with boric acid, the peak at 27.0 min reappeared and the shoulder at 27.8 min decreased slightly. The RG II monomers were esterified by borate to form the dimer again. The remaining peak at 27.8 min was probably another population of polysaccharides. However, for bilberry MHR it cannot be excluded that RG II was partly present in monomeric form, as well.

The pattern of black currant juice MHR showed a distinct peak at 27.8 min (figure 3.2). The conversion from dimer to monomer after acid treatment was, however, only visible by the disappearance of a shoulder at 27.0 min. This shoulder reappeared after incubation with boric acid. The difference between the shoulder at 27.0 min and the peak at 27.8 min is much smaller after acid treatment and incubation with boric acid compared to black currant MHR dissolved at pH 3.5. Thus, monomeric RG II might be present in black currant MHR, as well.

**Rhamnogalacturonan II is released by enzymatic degradation of pectin**

RG II is linked to pectin and can be release by enzymatic degradation of homogalacturonan (Darvill et al., 1978; Ishii and Matsunaga, 2001). Thus, AIS and the pectic fractions of the berries were incubated with homogalacturonan degrading enzymes to release RG II. Polygalacturonase (PG) combined with pectin methyl esterase (PME) or PG combined with pectin lyase (PL) were chosen to degrade methyl esterified and non-methyl esterified homogalacturonan.
Table 3.3  Sugar composition of bilberries and fraction derived from them analysed as TMS-derivatives using GC-FID [mol.%].

<table>
<thead>
<tr>
<th></th>
<th>2-O-methyl fuc</th>
<th>Rha</th>
<th>Fuc</th>
<th>2-O-methyl xyl</th>
<th>Ara</th>
<th>Api</th>
<th>Xyl</th>
<th>Man</th>
<th>Gal</th>
<th>Glc</th>
<th>GaLA</th>
<th>GlcA</th>
<th>AceA</th>
<th>DHA</th>
<th>KDO</th>
<th>total sugars [w/w%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIS</td>
<td>0.2</td>
<td>17.9</td>
<td>0.5</td>
<td>0.3</td>
<td>22.8</td>
<td>0.3</td>
<td>2.5</td>
<td>1.1</td>
<td>14.0</td>
<td>11.4</td>
<td>27.7</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
<td>0.2</td>
<td>11.9</td>
</tr>
<tr>
<td>HBSS</td>
<td>0.1</td>
<td>2.0</td>
<td>0.4</td>
<td>0.1</td>
<td>7.1</td>
<td>0.2</td>
<td>0.8</td>
<td>1.0</td>
<td>12.9</td>
<td>2.1</td>
<td>72.5</td>
<td>0.7</td>
<td>0.2</td>
<td>0.0</td>
<td>0.3</td>
<td>39.2</td>
</tr>
<tr>
<td>ChSS</td>
<td>0.0</td>
<td>1.0</td>
<td>0.2</td>
<td>0.0</td>
<td>3.1</td>
<td>0.1</td>
<td>0.7</td>
<td>0.3</td>
<td>7.8</td>
<td>2.6</td>
<td>83.2</td>
<td>0.6</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
<td>30.1</td>
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<tr>
<td>DASS</td>
<td>0.3</td>
<td>3.8</td>
<td>0.6</td>
<td>0.2</td>
<td>10.4</td>
<td>0.2</td>
<td>1.8</td>
<td>2.4</td>
<td>18.7</td>
<td>3.2</td>
<td>55.9</td>
<td>0.8</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>20.0</td>
</tr>
<tr>
<td>CASS</td>
<td>n.d.</td>
<td>2.3</td>
<td>0.3</td>
<td>n.d.</td>
<td>2.6</td>
<td>0.0</td>
<td>0.6</td>
<td>1.7</td>
<td>27.6</td>
<td>35.9</td>
<td>24.8</td>
<td>0.8</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>4.0</td>
</tr>
<tr>
<td>residue</td>
<td>0.4</td>
<td>26.0</td>
<td>0.4</td>
<td>0.5</td>
<td>33.3</td>
<td>0.1</td>
<td>1.8</td>
<td>1.0</td>
<td>8.8</td>
<td>12.1</td>
<td>14.8</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
<td>0.4</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Table 3.4  Sugar composition of black currants and fraction derived from them analysed as TMS-derivatives using GC-FID [mol.%].

<table>
<thead>
<tr>
<th></th>
<th>2-O-methyl fuc</th>
<th>Rha</th>
<th>Fuc</th>
<th>2-O-methyl xyl</th>
<th>Ara</th>
<th>Api</th>
<th>Xyl</th>
<th>Man</th>
<th>Gal</th>
<th>Glc</th>
<th>GaLA</th>
<th>GlcA</th>
<th>AceA</th>
<th>DHA</th>
<th>KDO</th>
<th>total sugars [w/w%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIS</td>
<td>0.6</td>
<td>5.1</td>
<td>0.8</td>
<td>0.7</td>
<td>17.0</td>
<td>0.7</td>
<td>1.4</td>
<td>14.1</td>
<td>7.8</td>
<td>29.0</td>
<td>0.9</td>
<td>0.9</td>
<td>0.7</td>
<td>0.6</td>
<td>0.0</td>
<td>23.8</td>
</tr>
<tr>
<td>HBSS</td>
<td>0.4</td>
<td>2.4</td>
<td>1.3</td>
<td>0.3</td>
<td>9.1</td>
<td>0.4</td>
<td>1.0</td>
<td>1.1</td>
<td>2.8</td>
<td>0.7</td>
<td>78.9</td>
<td>0.4</td>
<td>1.0</td>
<td>0.7</td>
<td>0.1</td>
<td>49.0</td>
</tr>
<tr>
<td>ChSS</td>
<td>0.3</td>
<td>1.9</td>
<td>0.5</td>
<td>0.2</td>
<td>10.2</td>
<td>0.2</td>
<td>2.5</td>
<td>0.4</td>
<td>4.4</td>
<td>0.5</td>
<td>77.0</td>
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<td>1.2</td>
<td>0.3</td>
<td>34.9</td>
</tr>
<tr>
<td>DASS</td>
<td>0.3</td>
<td>3.4</td>
<td>0.4</td>
<td>0.1</td>
<td>17.6</td>
<td>0.1</td>
<td>2.3</td>
<td>1.0</td>
<td>30.0</td>
<td>0.6</td>
<td>42.6</td>
<td>0.6</td>
<td>0.5</td>
<td>0.9</td>
<td>0.1</td>
<td>28.7</td>
</tr>
<tr>
<td>CASS</td>
<td>n.d.</td>
<td>3.2</td>
<td>0.5</td>
<td>n.d.</td>
<td>3.9</td>
<td>0.0</td>
<td>0.1</td>
<td>12.6</td>
<td>29.1</td>
<td>34.2</td>
<td>12.9</td>
<td>2.4</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>4.7</td>
</tr>
<tr>
<td>residue</td>
<td>0.1</td>
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<td>0.2</td>
<td>0.1</td>
<td>8.3</td>
<td>0.2</td>
<td>0.7</td>
<td>50.3</td>
<td>12.3</td>
<td>14.9</td>
<td>8.6</td>
<td>0.3</td>
<td>0.4</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>2-O-methyl fuc</th>
<th>Rha</th>
<th>Fuc</th>
<th>2-O-methyl xyl</th>
<th>Ara</th>
<th>Api</th>
<th>Xyl</th>
<th>Man</th>
<th>Gal</th>
<th>Glc</th>
<th>GaLA</th>
<th>GlcA</th>
<th>AceA</th>
<th>DHA</th>
<th>KDO</th>
<th>total sugars [w/w%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>juice MHR</td>
<td>0.5</td>
<td>9.0</td>
<td>0.6</td>
<td>0.4</td>
<td>20.9</td>
<td>0.5</td>
<td>0.5</td>
<td>0.8</td>
<td>6.2</td>
<td>7.2</td>
<td>50.4</td>
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<td>0.7</td>
<td>0.4</td>
<td>0.5</td>
<td>50.0</td>
</tr>
<tr>
<td>cakeAIS</td>
<td>0.1</td>
<td>4.4</td>
<td>0.7</td>
<td>0.2</td>
<td>9.8</td>
<td>0.2</td>
<td>0.7</td>
<td>38.8</td>
<td>13.4</td>
<td>13.0</td>
<td>16.8</td>
<td>0.4</td>
<td>1.2</td>
<td>0.4</td>
<td>0.0</td>
<td>23.1</td>
</tr>
</tbody>
</table>

n.d.: not detected
As an example, figure 3.3 shows the degradation of bilberry HBSS with PG/PME and PG/PL. After enzyme incubation a peak at 27.0 min appeared, representing dimeric RG II. Dimeric RG II was found after degradation of black currant HBSS and of bilberry and black currant AIS, as well. After pectin degradation in ChSS and DASS of bilberries and black currants, not only a peak at 27.0 min, but also a peak at 27.8 min appeared (data not shown), indicating the presence of monomeric and dimeric RG II. With HPSEC it was possible to estimate the contents of RG II by dividing the area of RG II (monomer and dimer) by the total area of degradation products (data not shown). Similar RG II contents were obtained as calculated via the content of 2-O-methyl fucose (table 3.1).

**Discussion**

Rhamnogalacturonan II (RG II) is known to be present in the cell walls of various angiosperms, as recently reviewed (O'Neill et al., 2004). We have shown that black currants and bilberries make no exception. All pectic fractions contain RG II, as shown by the presence of all its diagnostic sugar residues. RG II as a possible cross linker might influence the extractability of cell walls, which can be an important factor in juice processing. If RG II is present as a monomer, extraction of pectic polysaccharides is easier (Kakegawa et al., 2005). In ChSS

![Figure 3.1](image)

**Figure 3.1** HPSEC elution patterns of bilberry juice MHR a) in sodium acetate buffer pH 3.5, b) after treatment with 0.1 M HCl dialysed with sodium acetate buffer pH 3.5, and c) after treatment with 0.1 M HCl dialysed with sodium acetate buffer pH 3.5 containing 50 mM boric acid. Vertical lines indicate the elution time of RG II monomer and dimer.
Rhamnogalacturonan II is present in bilberries and black currants.

**Figure 3.2** HPSEC elution patterns of black currant juice MHR in a) sodium acetate buffer pH 3.5, a) after treatment with 0.1 M HCl followed by dialysis against sodium acetate buffer pH 3.5, and c) after treatment with 0.1 M HCl followed by dialysis against sodium acetate buffer pH 3.5 containing 50 mM boric acid. Vertical lines indicate the elution time of RG II monomer and dimer.

**Figure 3.3** HPSEC elution pattern of bilberry HBSS a) before and after incubation with b) PG/PME and c) PG/PL at pH 5.
and DASS of radish roots, RG II was identified and structurally analysed (Matoh et al., 1998). However, RG II was not quantified in cell wall material of bilberries and black currants before.

For quantification of RG II in different polysaccharide samples, TFA hydrolysis and derivatisation as alditol acetates is a suitable method. We were able to quantify RG II in pectin rich fractions, in juice and in press cake of bilberries and black currants, although the analysis was carried out close to the detection limit. The diagnostic acidic sugars present in RG II were identified after methanolysis of the different fractions followed by TMS derivatisation. This method is suitable for quantification of soluble material such as juice MHR. Compared to previous results, where Saeman-hydrolysis and derivatisation as alditol acetates was performed (Hilz et al., 2005), hardly any cellulose and hemicellulose could be determined.

In juice production, RG II is an important structural element because of its possible role as a cross linker of pectins and because of its enzyme resistance. By analysing juice MHR and cell wall polysaccharides in the press cake, we traced RG II during juice processing. In juice MHR of bilberries and black currants, RG II was the main polysaccharide and was present in higher relative amounts than in the cell wall fractions. During juice production pectinolytic enzymes are used to degrade pectins. Because the applied enzyme preparation was not able to degrade RG II, this pectic element accumulated and was extracted into the juice (about 60 % of total RG II). The press cakes contained still about 40 % of the RG II present in the berries. This RG II was, however, still linked to homogalacturonan and probably originated from the seeds, which ended up intact in the press cake. RG II accumulates in wine after enzymatic degradation of pectins during vinification, as well (Ayestarán et al., 2004). We showed that at least the major part of RG II is present as a dimer in juice, although berry juice is quite acidic (pH 2.8). Neither the applied enzyme preparation nor the acidity saponified the boron ester that cross-links the two RG II molecules.

By degradation with pectinolytic enzymes RG II was released from homogalacturonan in cell wall material and in all pectic fractions. This confirms that RG II is covalently linked to homogalacturonan (Ishii and Matsunaga, 2001: Reuhs et al., 2004). While RG II was released as only dimer from HBSS and AIS, RG II was released as both monomer and dimer from pectins present in ChSS and DASS. These fractions were treated with the chelating agent EDTA. EDTA forms a complex with calcium and removes calcium from the calcium-pectin complex. Insoluble, calcium sensitive pectins become soluble. Calcium was found to stabilise the RG II diester (Ishii et al., 1999; Kobayashi et al., 1999; Wimmer and Goldbach, 1999). With the help of chelating agents, monomeric RG II is formed from the dimer. EDTA is not able to complete this conversion (Ishii et al., 1999), whereas CDTA is (Matoh et al., 1998). Monomerisation of RG II is probably due to complexation of stabilising calcium and not due to interaction of chelating agent with the boron ester itself. The boron ester might help to retain chelating agent soluble pectins in the cell wall (Kobayashi et al., 1999). We suggested that the release of pectins with a high degree of methyl esterification
Rhamnogalacturonan II is present in bilberries and black currants (DM), thus calcium insensitive pectin, with chelating agent may partly be caused by hydrolysing the boron diester of RG II that is linked to homogalacturonan with a high DM (Hilz et al., 2005). Thus, the RG II monomers in ChSS and DASS of bilberries and black currants were probably formed during extraction. However, the possibility that the monomeric form is present in muro cannot be ruled out.

The results of this study may indicate that RG II is an important polysaccharide for the juice industry. It is present in all pectic population of bilberries and black currants, in muro probably always in the dimeric form. It might cross-link two pectin molecules and, therefore, improve gel formation, which hinders the processability of berries. Furthermore, RG II is released from pectin molecules and is accumulated in the juice during processing. Enzymatic degradation of the covalent, pectic RG II might enable the production of special fruit juices.

Acknowledgment

We thank Prof. Kaisa Poutanen and Mirja Mokkila, VTT Biotechnology, Finland, for coordinating the MAXFUN project and Vernu Vasunta, Kiantama Ltd., Finland, for providing us with commercial berry material.

This study has been carried out with financial support from the Commission of the European Communities, specific RTD programme ‘Quality of Life and management of Living Resources’, contract number QLK1-CT-2002-02364 ‘Novel enzyme-aided extraction technologies for maximized yield and functionality of bioactive components in consumer products and ingredients from by-products’, acronym MAXFUN. It does not necessarily reflect its views and in no way anticipates the Commission’s future policy in this area.
Chapter 4

A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants

Published as
Abstract

Different separation (HPAEC, RP-HPLC, CE) and identification (MALDI-TOF MS, ESIMS\textsuperscript{n}) techniques were compared to analyse oligosaccharides obtained after incubation of xyloglucan with endo-glucanase. It was possible to analyse xyloglucan oligosaccharides with each technique. Several techniques, including off line (HPAEC-MALDI-TOF MS) or online (CE-ESIMS\textsuperscript{n}, RP-HPLC-ESIMS\textsuperscript{n}) connection provided complementary information on xyloglucan structure. Online CE-MS and RP-HPLC-MS are described for the first time in xyloglucan analysis. Advantages and disadvantages of the techniques for different purposes such as structural characterisation of oligosaccharides or oligosaccharide profiling are discussed. Black currant xyloglucans had rather simple XXXG-type structure with galactose and fucose containing side chains.

Keywords
Ribes nigrum; black currant; cell wall polysaccharides; xyloglucan; CE-MS; HPLC-MS; HPAEC; MALDI-TOF MS
Introduction

Xyloglucans belong to the group of hemicelluloses and were first described in tamarind seeds, where they function as storage polysaccharides (Savur and Sreenivasan, 1948). Later xyloglucans were also described in plant cell walls (Bauer et al., 1973) and since then their structure and the role in the plant cell wall have been studied intensively. Today it is known that xyloglucan is the main hemicellulose of dicotyledons and its composition is dependent on the taxonomic family (Vierhuis et al., 2001; Hoffman et al., 2005).

Xyloglucans cover and interlink cellulose microfibrils (Keegstra et al., 1973; Hayashi et al., 1987). In the plant cell wall’s cellulose-xyloglucan network three xyloglucan domains were described: one domain is present in free loops or cross links and can be degraded by endo-glucanases, a second domain covers the cellulose microfibrils and can be extracted with concentrated alkali, and a third domain is entrapped within the amorphous cellulose microfibrils and can only be accessed after degradation of cellulose (Hayashi et al., 1987; Pauly et al., 1999a; Bootten et al., 2004).

![Figure 4.1 Xyloglucan oligomer XLFG as example for XXXG-type xyloglucan.](image)

The backbone of xyloglucan is a β-1,4-linked glucan chain (Aspinall and Molloy, 1969) to which different short side chains are attached (Bauer et al., 1973). To simplify the nomenclature, the different side chains of xyloglucan were assigned one-letter codes such as G for unsubstituted glucose and X for a xylose attached to the glucose (Fry et al., 1993). Xyloglucan can be classified in XXXG-type (figure 4.1), XXGG-type (typical for Solanaceae), and XXXGG-type structures (Buckeridge et al., 1997; Vincken et al., 1997). XXXX-type xyloglucan of which every glucose is substituted with arabinose in position 2 was also reported (Mabusela et al., 1990). Molecular modelling showed that the fucose-galactose containing side chain (F) is important for flattening of the backbone in solution and, therefore, initiates xyloglucan-cellulose binding (Levy et al., 1991). However, fucose is not required for cellulose binding (Hanus and Mazeau, 2006).

Black currants are an important crop of northern Europe and are mainly used for juice production. In our recent studies, cell wall polysaccharides of black currants were characterised in detail (Hilz et al., 2005; Hilz et al., 2006b). The hemicellulose rich fraction of black currants contained a high amount of xylose and glucose, indicating the presence of xyloglucan. The structure of xyloglucan is regular and similar within one botanic family (Hoffman et al., 2005).
information of black currants was obtained from the NCBI database (Wheeler et al., 2000). Black currants belong to the family of Grossulariaceae, order Saxifragales. The structure of xyloglucan of neither a member of the same family nor of the same order has been reported in literature until now.

High performance anion exchange chromatography (HPAEC) is the most common technique for separation of xyloglucan oligomers (Vincken et al., 1996). Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) together with post source decay (PSD) was used for determination of the mass and structure of different oligomers (York et al., 1995; Vierhuis et al., 2001; Bauer et al., 2005; Hoffman et al., 2005). While oligosaccharides were derivatised to their acetylated form and analysed by fast-atom bombardment mass spectrometry (FAB-MS) in the past (York et al., 1988), the development of improved ionisation techniques and more sensitive electrospray ionisation mass spectrometers (ESIMSα) made it possible to use MS and MSn of underivatised oligomers to unravel their structure (Quemener et al., 2003). Instead of HPAEC, reversed phase high performance liquid chromatography (RP-HPLC) has also been used for determination of xyloglucan oligomers (Pauly and York, 1998) allowing online ESIMSα-detection. Recently capillary electrophoresis (CE) of labelled xyloglucan oligomers was performed and the peaks were identified by comparing them to isolated fractions from HPAEC (Bauer et al., 2005). To prove the presence of xyloglucans and to characterise them, xyloglucans of black currants were degraded by xyloglucan specific endo-glucanase (XEG) and the obtained oligomers were analysed using a number of different techniques.

The aim of this study was to give an overview of state of the art techniques that are suitable for xyloglucan analysis. Therefore xyloglucan is degraded by endo-glucanase and the obtained oligomers are analysed for oligosaccharide profiling and structural characterisation of the oligosaccharides. The advantages and disadvantages of the different techniques for these two purposes are exemplified on black currant xyloglucans, which have not been characterised before.

Materials and Methods

Material

Black currants (Ribes nigrum L.) were obtained from Kiantama Ltd., Suomussalmi, Finland. Alcohol insoluble solids (AIS) were prepared from homogenised berries. In 3 extraction steps the pectic polysaccharides were extracted from AIS. The hemicelluloses were extracted from the residual cellulose with 6 M sodium hydroxide (concentrated alkali soluble solids; CASS). The solution was directly neutralised in an ice bath, subsequently dialysed and freeze dried (Hilz et al., 2005).

Fractionation of hemicelluloses with anion exchange chromatography

About 250 mg CASS were dissolved in water and centrifuged. The pellet was collected (CASS residue) and the supernatant was fractionated on a DEAE
Sepharose Fast Flow column (100 x 2.6 cm, GE Healthcare, Upsala, Sweden) into
three fractions: unbound material was eluted with 530 mL 5 mM sodium acetate
at pH 5.0 (CASS unbound), the second fraction was eluted with 530 mL 2 M
sodium acetate buffer at pH 5.0 (CASS NaOAc), and the last fraction was eluted
with 530 mL 0.5 M sodium hydroxide (CASS NaOH) (Huisman et al., 2000).

**Degradation of xyloglucan with xyloglucan specific endo-glucanase (XEG)**

Xyloglucan containing material (5 mg) was dissolved in 1 mL 50 mM sodium
acetate buffer (pH 5.0) and incubated with 1 µL xyloglucan specific endo-
glucanase (XEG, EC 3.2.1.4 from *Aspergillus aculeatus*, 2259 U/mL) over night.
AIS (5 mg/mL) were incubated with additional 5 µL polygalacturonase
(EC 2.1.1.15 from *Kluyveromyces fragilis*, 16 U/ml) and 1 µL pectin methyl
esterase (EC 3.1.1.11 from *Aspergillus niger*, 180 U/ml) for pectin degradation
(Vierhuis et al., 2001).

**Sugar composition as alditol acetates**

For determining the sugar composition, samples were pre-hydrolysed using
72 % w/w sulphuric acid at 30 °C for 1h and subsequently hydrolyzed with 1 M
sulphuric acid at 100 °C for 3 h (Saeman et al., 1945). Afterwards the sugars
were derivatised to their alditol acetates and determined by gas chromatography
(Englyst and Cummings, 1984) using inositol as internal standard.

**Uronic acid content.**

The total uronic acid content was determined photometrically with the
automated m-hydroxydiphenyl assay (Thibault, 1979).

**HPAEC of xyloglucan oligomers**

Xyloglucan oligosaccharides were analysed on a CarboPac PA 100 column (4 x
250 mm, Dionex, Sunnyvale, USA) and on a CarboPac PA 1 column (2 x 250 mm,
Dionex, Sunnyvale, USA) with pulse amperometric detection (PAD) using a
column specific sodium hydroxide/sodium acetate-gradient as described before
(Vincken et al., 1996) and a flow of 1 mL/min for the 4 x 250 mm column and of
0.3 mL/min for the 2 x 250 mm column, respectively.

Desalted fractions were obtained in two ways:

a) The ultra-self-regenerating anion suppressor 4 mm-unit (ASRS, Dionex,
Sunnyvale, USA) was connected after the PAD to exchange the sodium ions for
hydronium ions (H$_3$O$^+$) followed by the ultra-self-regenerating cation suppressor
4 mm-unit (CSRS, Dionex, Sunnyvale, USA) to exchange the acetate ions for
hydroxide ions (OH$^-$). The continuous desalting of the eluent was achieved by the
electrolysis of demineralised water (8 ml/min) in both suppressors. Fractions of
30 s were collected in a 96-well plate using a FC-203B fraction collector (Gilson,
Middleton, USA) and freeze dried (Kabel et al., 2001).

b) Directly after the PAD the FC-203B fraction collector (Gilson, Middleton,
USA) was connected collecting fractions of 30 s. These fractions were collected in
a Sep-Pak tC18 (40 mg) 96-well plate (Waters, Milford, USA) containing 0.5 mL
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0.5 M acetic acid per well. The eluent was sucked through the Sep-Pak tC18 (40 mg) 96-well plate by vacuum with a MultiScreen resist vacuum manifold (Millipore, Billerica, USA). After washing 2 times with 1 mL water, the oligomers were eluted with 2 mL methanol and dried at 60 °C.

**MALDI-TOF MS**

Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) was performed using an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm and operated in positive mode. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12000 V. The ions were detected using reflector mode. The lowest laser power required to obtain good spectra was used and at least 100 spectra were collected. The mass spectrometer was calibrated with a mixture of maltodextrins (mass range 300-2500).

2 µL sample solution were mixed on the MALDI-TOF-plate (Bruker Daltonics, Bremen, Germany) with 2 µL matrix solution of 9 mg/mL 2,5 dihydroxybenzoic acid (Bruker Daltonics, Bremen, Germany) in 30 % acetonitrile and dried under a stream of air (Verhoef et al., 2005).

**Post-source decay**

For post source decay (PSD) the same conditions as for MALDI-TOF MS were used. PSD was recorded in the positive mode. The spectrum was dived into different independently measured segments, which were put together to one PSD spectrum by the software FlexAnalysis (Bruker Daltonics, Bremen, Germany) (Verhoef et al., 2005).

**ESIMS**

Electrospray ionisation mass spectrometry (ESIMS) was performed on a LTQ Ion-trap (Thermo Electron, San Jose, USA). The sample was applied through a PicoTip emitter capillary (4 µm ID of the tip, New Objective, Woburn, USA). MS analysis was carried out in the positive mode using a spray voltage of 1.5 kV and a capillary temperature of 200 °C, auto-tuned on xyloglucan oligosaccharides. MS² and higher was performed using a window of 1 m/z and a relative collision energy of 30 %.

**RP-HPLC-ELSD-ESIMS**

Xyloglucan oligomers were separated by reversed phase HPLC (RP-HPLC) on a XTerra MS C18 3.5 µm column (4.6 x 150 mm, Waters, Milford, USA) with a linear gradient from 0 to 15 % of methanol in 100 min followed by a 10 min washing step with 100 % methanol at a flow of 0.9 mL/min. After post column splitting 3/4 of the eluent went to a evaporate light scattering detector ELSD 2000 (Alltech, Lexington, USA) and 1/4 went to a LCQ Deca XP MAX ESIMS detector (Thermo Electron, San Jose, USA). ESIMS was operated in positive mode using a spray voltage of 4.0 kV and a capillary temperature of 200 °C and
auto-tuned on xyloglucan oligosaccharides. MS² and higher was performed using a window of 1 m/z and a relative collision energy of 30 %.

**CE-LIFD**

For capillary electrophoresis (CE) xyloglucan oligosaccharides were labelled with 8-aminoperylene-1,3,6-trisulfonate (APTS) using the ProteomeLab Protein Characterisation kit (Beckman Coulter, Fullerton, USA).

The labelled oligosaccharides were separated on a polyvinyl alcohol (N CHO) coated capillary (50 µm ID x 50.2 cm, detector after 40 cm, Beckman Coulter, Fullerton, USA) using a ProteomeLab PA 800 characterisation system (Beckman Coulter, Fullerton, USA) and a laser induced fluorescence detector (LIFD) at an excitation of 488 nm and an emission of 520 nm (Beckman Coulter, Fullerton, USA). The separation was carried out in reversed polarity at 30 kV in a 25 mM sodium acetate buffer containing 0.4 % polyethylene oxide at pH 4.75. The capillary was kept at 25 °C. APTS labelled maltose was used as internal standard.

**CE-ESIMS**

For identification of the different oligomers, separation was carried out on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) with a fused silica capillary (50 µm ID x 80 cm) connected to a UV-detector and an ESIMS detector (LTQ Ion-trap, Thermo Electron, San Jose, USA) by a device made in our laboratory (figure 4.2). The separation was carried out at reversed polarity at 28 kV in a 50 mM ammonium acetate buffer containing 0.2 % formic acid at pH 2.8. The capillary was kept at 15 °C. For ESIMS a sheath flow of 4 µL/min of 75 % isopropanol in water was used. ESIMS was operated in the negative mode using a spray voltage of 2.2 kV and a capillary temperature of 190 °C and auto-tuned on xyloglucan oligosaccharides. MS2 and higher was performed using a window of 1 m/z and a relative collision energy of 30 %.

![Figure 4.2 Schematic view of CE-MS device.](image-url)
Results and Discussion

Xyloglucans from black currants were analysed following two different approaches. In the first approach, hemicelluloses were extracted with 6 M sodium hydroxide from cellulose microfibrils after removal of pectins (concentrated alkali soluble solids; CASS). Xyloglucans were further purified on an anion exchange column. Neutral hemicelluloses including xyloglucan were separated from anionic hemicelluloses and pectins. However, possible acetyl esters were saponified during alkali extraction and could not be determined by this approach. Thus, to analyse a possible acetylation pattern of xyloglucan, xyloglucans were degraded with xyloglucan-specific endo-glucanase (XEG) directly in alcohol insoluble solids (AIS) in a second approach (Vierhuis et al., 2001).

Yield and sugar composition of xyloglucan containing fractions

Sugar compositions of cell wall fractions indicate the polysaccharides present. In CASS xylose, glucose, and mannose were the major sugar residues (table 4.1). The high amount of xylose present in a 1.6:1 ratio to glucose indicated the presence of xylans next to xyloglucans. Uronic acid was present in small amounts. These uronic acids can be glucuronic acids from glucuronooarabinoxylans or galacturonic acids from pectins (Hilz et al., 2005). CASS were hardly soluble in water: 83 % remained insoluble (CASS residue). The dissolved polysaccharides were loaded on an anion exchange column. The smallest part of them did not bind to the column and was eluted with water (CASS unbound). More polysaccharides were eluted with 2 M sodium acetate (CASS NaOAc) and 0.5 M sodium hydroxide (CASS NaOH). CASS unbound contained xylose and glucose in a ration 2:4. The amounts of mannose and galactose were also high showing that next to xyloglucans galactomannans were present (Hilz et al., 2005). The high amount of fucose suggested a high percentage of fucosilated xyloglucan (vide infra). In CASS NaOAc and CASS NaOH, xylose was by far the major sugar moiety. High amounts of uronic acids and high amounts of arabinose and mannose were present, as well. CASS NaOH and CASS NaOAc differed in the content of glucose: while CASS NaOAc contained only 5 mol%, CASS NaOH contain 10 mol%. These two fractions were rich in charged xylans and contained some galactomannans and maybe pectins. CASS residue contained xylose and glucose in a ratio of 2.4:4 accompanied by

<table>
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<th>Table 4.1 Sugar composition of xyloglucan containing fractions of black currants [mol %].</th>
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<td>Rha</td>
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<tr>
<td>CASS total</td>
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<td>CASS unbound</td>
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<tr>
<td>CASS NaOAc</td>
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<td>CASS NaOH</td>
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<td>CASS residue</td>
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<td>AIS</td>
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60
Chapter 4

fucose. Additionally, mannose and galactose residues were present. Mainly xyloglucan and galactomannan and maybe some xylans were present in the CASS residue. AIS of black currants contained mannose, glucose, and uronic acid as major sugar residues. Xylose was present in minor quantities.

The main hemicelluloses present in black currants were xyloglucans, xylans, and galactomannans. However, sugar composition can only indicate the presence of these polysaccharides, but for proving the presence of the different hemicelluloses other analytical approaches including enzyme degradation are necessary.

**HPAEC**

Endo-glucanase II is a xyloglucan specific endo-glucanase (XEG), which is known to split xyloglucan between an unsubstituted glucose and a xylose-substituted glucose residue (Pauly et al., 1999b). By this digestion xyloglucan can be degraded into specific oligosaccharide building blocks of xyloglucan (Fry et al., 1993).

The chromatograms of black currant xyloglucan oligomers separated on two different HPAEC columns are shown in figure 4.3. The different oligomers were identified by comparing the retention time with other known xyloglucan oligosaccharides from tamarind (Vincken et al., 1996) olive (Vierhuis et al., 2001) and soy (Huisman et al., 2000) and, additionally, by off-line MALDI-TOF MS and ESIMS. For MALDI-TOF MS and ESIMS sample solutions were desalted. In one desalting procedure tested, two desalting membranes were connected after the detector to remove anions and cations from the eluting fractions (Kabel et al., 2001). With this method it was possible to identify the peaks in the elution pattern using MALDI-TOF MS and ESIMS, but peak broadening was observed and the retention time from the PAD was delayed because of tubing dead volume of the membrane devices. These problems were solved in the second desalting procedure by using a C18-solid phase extraction (SPE) 96-well plate for fraction collection. The fractions were collected directly after the PAD. After SPE, the final fractions were salt free and, additionally, peak broadening was prevented. By identifying the peaks it was shown that the CarboPak PA 1 column separated XXLG from XLFG, but not XLXG and XLFG, the CarboPak PA 100 column separated XLXG and XLFG, but not XXLG and XLFG.

Black currant xyloglucans consisted of three main and four minor oligomers. Xyloglucan oligomers of black currants carry galactose (L) and fucose-galactose (F) containing side chains. Only by using the two different HPAEC columns and off-line ESIMS, it was possible to show that both XLXG and XXLG were present. Xyloglucan oligomers were shown in XEG digests of both the unbound (neutral) and bound (charged) fractions obtained from anion exchange chromatography of CASS, although neutral polysaccharides such as xyloglucans should not bind to an anion exchanger. This behaviour was shown before and was explained by a proposed covalent cross link of the reducing glucose of xyloglucan to pectic galactan (Keegstra et al., 1973).
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Figure 4.3 Xyloglucan oligomers obtained after degradation with XEG on HPAEC with a 2 mm Dionex PA1 (A) and a 4 mm Dionex PA100 (B) column.

Relative amounts of the different xyloglucan oligomers in the hemicellulose containing fractions are shown in table 4.2. CASS, CASS unbound, CASS NaOAc, CASS NaOH, and CASS residue showed no significant differences. XLFG was the major oligomer followed by XXXG and XXFG. In the xyloglucan oligomers derived from AIS more minor oligomers were present compared to digests of CASS and fractions derived from them. Although the solubility and elution behaviour on anion exchange chromatography of xyloglucans extracted with 6 M alkali (CASS and fractions derived from them) was different, the relative amounts of oligomers present were the same. Probably not the structure of xyloglucan determines the elution behaviour, but the binding of xyloglucan to other (charged) polysaccharides like xylans or pectins. This is in agreement with a proposed covalent cross link between xyloglucans and pectins (Keegstra et al., 1973; Thompson and Fry, 2000; Popper and Fry, 2005).

The relative proportions of the different xyloglucan oligomers differed in black currant AIS compared to CASS and the fractions derived from it. After degradation of xyloglucan inAIS, XXXG was the main oligomer, followed by XLFG and XXFG in the same amounts. XLXG, XXLG, and XLLG were minor oligomers. The accessibility of xyloglucan can explain these findings. In AIS only the xyloglucan that cross links celluloses or forms free loops is available for XEG (Pauly et al., 1999a). Another xyloglucan domain is still attached to cellulose and cannot be degraded in AIS, but will be extracted with concentrated alkali (Pauly et al., 1999a). A possible acetylation of the galactose unit as shown in sycamore
Table 4.2 Oligomers present in xyloglucan of black currants as calculated from HPAEC with a Dionex PA1 (A) and a Dionex PA 100 (B) column.

<table>
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<tr>
<td></td>
<td>XXG</td>
<td>XXXG</td>
<td>XXFG</td>
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<td>XXLG</td>
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<td>[Area %]</td>
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<tr>
<td>CASS total</td>
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<td>26</td>
<td>-</td>
<td>37</td>
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<td>31</td>
<td>27</td>
<td>-</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>CASS NaOAc</td>
<td>1</td>
<td>30</td>
<td>24</td>
<td>-</td>
<td>40</td>
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<td>CASS NaOH</td>
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<td>31</td>
<td>26</td>
<td>-</td>
<td>39</td>
<td>2</td>
</tr>
<tr>
<td>CASS residue</td>
<td>2</td>
<td>32</td>
<td>26</td>
<td>-</td>
<td>36</td>
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<td>3</td>
<td>34</td>
<td>28</td>
<td>-</td>
<td>30</td>
<td>5</td>
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<th>B PA 100</th>
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<tr>
<td></td>
<td>XXG</td>
<td>XXXG</td>
<td>XXFG</td>
<td>XLXG</td>
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<td>XXLG</td>
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<td>[Area %]</td>
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<td>CASS total</td>
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<td>CASS unbound</td>
<td>2</td>
<td>32</td>
<td>26</td>
<td>2</td>
<td>37</td>
<td>-</td>
</tr>
<tr>
<td>CASS NaOAc</td>
<td>3</td>
<td>29</td>
<td>24</td>
<td>2</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>CASS NaOH</td>
<td>2</td>
<td>31</td>
<td>25</td>
<td>2</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>CASS residue</td>
<td>4</td>
<td>23</td>
<td>28</td>
<td>6</td>
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</tr>
<tr>
<td>AIS</td>
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<td>33</td>
<td>27</td>
<td>5</td>
<td>27</td>
<td>-</td>
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xyloglucan (Kiefer et al., 1989) might be another explanation for a difference in degradability of xyloglucan domains. Acetylation of the galactose residues of xyloglucans in AIS might partly hinder enzymatic degradation of cellulose bound xyloglucan. However, only acetylation of glucose residues of the backbone inhibits degradation by XEG (York et al., 1996).

HPAEC is a robust method to analyse oligosaccharide profiles of xyloglucan. The possibility to couple HPAEC off line to mass spectrometers allows identification of unknown oligomers. Especially the use of SPE for desalting simplifies the off line coupling. A disadvantage is the necessity for two different columns to separate all oligomers and difficult absolute quantification due to different response factors by PAD of mono and oligomers (Hotchkiss and Hicks, 1990).

**MALDI-TOF MS**

Oligosaccharides obtained by degradation of xyloglucan with XEG can be quickly identified by the mass to charge ratios of their sodium adducts with MALDI-TOF MS. A comparison of saponified oligomers, as obtained from the alkali extract, with non-saponified oligomers, as obtained from AIS, shows acetylation of oligomers by a m/z difference of 42 to the oligomer without an acetyl group. Prior to analysis, solid phase extraction (SPE) can be used to desalt the enzyme digest of xyloglucans and to remove pectic oligomers from it (Vierhuis et al., 2001). This is an elegant way to remove interfering charged oligosaccharides.

The mass spectrum of xyloglucan oligosaccharides derived from CASS showed three major and two minor peaks (Figure 4.4). The three major peaks belonged to the single charged sodium adducts of XXXG (m/z = 1085), XXFG (m/z = 1393), and XLFG (m/z = 1555). These oligomers were present in the highest amounts as determined by HPAEC. Small peaks of potassium adducts were present for all
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**Figure 4.4** MALDI-TOF MS spectrum of xyloglucan oligomers obtained after XEG treatment of black currant CASS (only sodium adducts are labelled).

**Figure 4.5** MALDI-TOF MS spectrum of xyloglucan oligomers obtained after XEG treatment of black currant AIS (only sodium adducts are labelled).
oligomers, even the minor ones. The m/z of 1409 is the m/z of either the sodium adduct of XLLG or the potassium adduct of XXFG. Probably both were present, but by comparing the ratio of sodium to potassium adducts of the other oligomers it can be concluded that the major part of the peak was the sodium adduct of XLLG. The other small peak was attributed to XLXG or XXLG (m/z = 1247). However, a distinction of the two oligomers with the same mass is not possible by MALDI-TOF-MS.

Comparing the mass spectrum of xyloglucan oligosaccharides derived from CASS (figure 4.4) to the mass spectrum of the ones derived from AIS (figure 4.5), six additional peaks were present in the mass spectrum of AIS. These oligomers carried one (m/z = 1289, m/z = 1435, m/z = 1451, m/z = 1597) or two (m/z = 1493, m/z = 1639) acetyl groups. Only the galactose containing oligosaccharides were acetylated. The oligosaccharides that carry two galactose residues (XLLG, XLFG) contained even two acetyl groups. Acetylation was reported to occur on the galactose residue of xyloglucan (York et al., 1988; Kiefer et al., 1989). Other possible binding sites for acetyl groups are arabinose units or O6 of a glucose unit in XXGG-type xyloglucans (Sims et al., 1996), but those kinds of oligomers were not present in black currant xyloglucan.

MALDI-TOF MS is a fast technique for oligosaccharide profiling and for demonstrating the presence of acetylated oligomers. However, oligomers having the same molecular mass are not distinguished. In time consumption and handling MALDI-TOF-MS outclasses ESI-MS, but structural characterisation of oligomers including localising of acetyl groups is not possible.

**MALDI-TOF MS-PSD**

To identify the positions of the different side chain and of the acetylated residues in the xyloglucan building blocks, post source decay (PSD) provides more structural information (Vierhuis et al., 2001).

From black currant AIS a xyloglucan oligomer with a mass of an acetylated XXFG (sodium adduct m/z = 1435) was released as shown by MALDI-TOF-MS (figure 4.5). The PSD-spectrum and the structure of this oligomer are shown in figure 4.6. The asterisk (*) marks the reducing end of this oligomer, which is cleaved from the oligomer always in the hydrated form (m = 180 Da). The peak with the highest intensity corresponded to the mass of the oligomer without the fucose unit (m/z = 1289). This excluded already acetylation of the fucose residue in XXFG, although a fragment of XXFG without fucose and acetyl was shown (m/z = 1247). Thus, the acetyl group was able to leave the molecule independently. The second peak in intensity (m/z = 1085) corresponded to the oligomer without fucose and an acetylated hexose. The only non-reducing hexosethat can leave the oligomer after the fucose is removed is the galactose residue. Therefore, this mass to charge ratio of 1085 indicated that the galactose was acetylated, although a fragment without a pentose and an acetyl group (m/z = 1261) that would indicate acetylation of a xylose was shown in lower intensity. The fragments with m/z = 805 and m/z = 847 corresponded to the FG*
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Figure 4.6 Post source decay (PSD) spectrum of acetylated XXFG (m/z = 1435, structure is shown in the left top corner).

and acetylated FG*, respectively, and confirmed the position of the F side chain next to the reducing end. In addition, the fragment with m/z = 847 showed that the acetyl group was present on the FG* fragment. Since acetylation on the reducing glucose would inhibit enzymatic cleavage of xyloglucan at this position (York et al., 1996), acetylation of the galactose unit was most probable, but could not be proven by PSD.

PSD spectra of all acetylated oligomers indicated acetylation of galactose, as known for xyloglucans from sycamore cells (York et al., 1988; Kiefer et al., 1989). Double acetylation of one residue as reported for extracellular xyloglucan of sycamore cells (York et al., 1988) was not seen. Furthermore, the m/z = 1247 peak was identified as a mixture of XXLG and XLXG and the structure of XLFG was confirmed with PSD, as well.

In xyloglucan analysis PSD can be used for identification of the side chain position of xyloglucan oligomers. However, definitive confirmation of the position of the acetyl group is difficult. The background noise is high and the accuracy of mass to charge ratios is not always optimal. Therefore, ESIMS* of the oligomers was performed to confirm the results of PSD, to prove the position of the acetyl groups, and to compare the two techniques.

ESIMS*

Alternatively to PSD, electrospray ionisation mass spectrometry (ESIMS) with ion trap is an opportunity to fragment selected ions and to get an insight in the structure of xyloglucan oligomers. ESIMS* is applicable for both, mixtures and
fractions derived from the analytical HPAEC after SPE. Mild fragmentation conditions were used to obtain glycosidic cleavages rather than cross ring cleavages.

The ESIMS$^2$ spectrum of acetylated XXFG (m/z = 1435) showed less background noise and better resolved peaks (figure 4.7) compared to the PSD spectrum of the same oligomer (figure 4.6). The sensitivity was much higher, so that a 50 times zoom in the lower m/z region below m/z = 1000 showed still very well resolved peaks and hardly any background noise.

When the mother ion of acetylated XXFG (m/z = 1435) was fragmented, the most apparent peak corresponded to the oligomer without fucose as already seen with PSD. Other peaks present proved that the acetyl group was present at the galactose unit. Only when the galactose unit was cleaved off the acetyl group was removed, as well. When a deoxy hexose and a hexose left the oligomer, the acetyl group left as well (m/z = 1085). However, when a deoxy hexose was cleaved together with a pentose, the acetyl group remained at the main ions (m/z = 1157).

With ESIMS$^2$ the acetyl group did not leave the oligomer independently from the sugar residue, as it was the case for PSD. The removal of fucose and xylose at the same time (fragment m/z = 1157) can only be explained by a double cleavage in the xyloglucan oligomer: The fragment m/z = 847 in MS$^2$ spectrum of m/z = 1435 together with the same fragment without fucose (m/z = 701) in the MS$^3$ spectrum of the defucosilated oligomer (m/z = 1289) showed fucosilation of this fragment. The MS$^4$ spectrum of the m/z = 701 fragment showed the removal of acetylated galactose (remaining fragments m/z = 497), but no xylose (theoretical m/z = 569). Therefore, fucose can only be attached to galactose, as reported before (Wilder and Albersheim, 1973; York et al., 1990), and not to xylose.

With ESIMS$^2$ it was possible to identify the galactose residue of xyloglucan oligomers as carrier of the acetyl groups. Double acetylation on one galactose unit as seen in extracellular xyloglucan of sycamore cells (York et al., 1988) was not found in black currant xyloglucan. The double acetylated oligomer of XLFG (m/z = 1639) carried the acetyl groups on two different galactose units. Furthermore, the structures of the xyloglucan oligomers XXXG, XXFG, XLFG, XLLG, and a mixture of XLLG and XXLG were confirmed.

ESIMS$^2$ with ion trap is a fast and sensitive method for the determination of oligosaccharide structures. It is more sensitive than PSD and for oligomers of which MS$^2$ is ambiguous MS$^n$ can be performed, depending on the concentration to MS$^6$ or MS$^7$. Especially the low background noise, which allows identification and further fragmentation of m/z peaks with a sensitive device even in 50 times zoom, makes ESIMS$^n$ superior of MALDI-TOF MS-PSD.

**RP-HPLC**

An alternative to HPAEC is reversed phase HPLC (RP-HPLC). Xyloglucan oligosaccharides derived from CASS can be separated. The advantage of RP-HPLC is the online connection to a mass spectrometer parallel to an evaporate light scattering detector (ELSD). A desalting procedure is not necessary.
A comparison of methods to determine xyloglucan structures in black currants

Figure 4.7 ESIMS²-spectrum of acetylated XXFG oligomer (structure is shown in the left top corner).

In the chromatogram of xyloglucan oligosaccharides derived from CASS is shown in figure 4.8. Four xyloglucan oligomers were identified by online ESIMS and ESIMS². These oligomers were also identified by HPAEC (figure 4.3). RP-HPLC separated the alpha and beta anomers of each oligomer (Pauly and York, 1998) so that the chromatogram showed two peaks with the same mass. The area percentage differed from the area percentage of the HPAEC, although the general tendency with XLFG being the most apparent oligosaccharide in xyloglucan oligosaccharides derived from CASS was the same for both techniques. While the differences between the areas of the XXXG, XXFG, and XLFG peaks were only small compared to those on HPAEC, the area of XLFG (57 %) was more than double of the other two main oligosaccharides XXXG (21 %) and XXFG (20 %) with RP-HPLC. This may be partly due to different response factors of the ELSD to the HPAEC-PAD (Hotchkiss and Hicks, 1990). The oligomer XLLG was not detected by this RP-HPLC-MS and the oligomers XXLG and XLXG were not separated. RP-HPLC is an alternative to HPAEC for separation and online identification (ESIMS²) of different xyloglucan oligomers. However, our RP-HPLC system did not allow a faster or better separation than HPAEC. Not all oligomers were detected and separation of alpha and beta anomers makes the chromatograms more complicated. When an HPAEC system is available, it should be used preferably.
Figure 4.8 RP-HPLC elution pattern of xyloglucan oligosaccharides derived from CASS.

CE-LIFD and CE-MS

Recently capillary electrophoresis (CE) became of interest in the carbohydrate analysis (Evangelista et al., 1995). Advantages of CE are short measuring times and good resolution of the different compounds, while hardly any solvents are needed. However, labelling of neutral oligosaccharides with for example APTS is necessary (Evangelista et al., 1995). With APTS three charges are introduced, which allow the neutral oligomers to migrate under electrophoretic conditions. Furthermore, APTS is fluorescent and allows laser induced fluorescence detection (LIFD) of the labelled sugars. By using an internal standard, quantification of the different oligomers is possible.

The electropherograms of APTS labelled xyloglucan oligosaccharides derived from black currant CASS and AIS are shown in Figure 4.9. Components migrating to the detector between 3 and 4 min were degradation products of the label (APTS). After 4.9 min the internal standard maltose and between 6 and 8 min the xyloglucan oligomer peaks were detected. The electropherogram of xyloglucan oligosaccharides derived from AIS differed from the one derived from CASS in having a double peak instead of a single peak at the last oligomer peak. Identification of the peaks and, therefore, the electrophoretic mobility of the different labelled oligomers was not possible by LIFD.

When the CE was, however, coupled online to an ESIMS detector, the labelled oligomers were identified by their mass to charge ratios. The conditions for CE-MS were modified to obtain the same order of migration by using a longer capillary that had no polyvinyl alcohol coating compared to CE-LIFD. To
suppress the stronger electro osmotic flow in the capillary, the buffer pH was lowered. Due to the introduction of three negative charges with the APTS-label, the oligomers were mainly detected as triple charged ions. The mass to charge ratios are shown in the CE-MS detected electropherogram (figure 4.10), which showed only a slight decrease in resolution compared to CE-LIFD. The time of the measurement was nearly doubled due to a longer capillary used to couple CE and ESIMS. We even managed to obtain high quality MS2 spectra of the CE separated, APTS labelled oligomers (figure 4.10).

The smallest oligomers had the highest electrophoretic mobility and were detected first, while the largest oligomers were detected last due their lower electrophoretic mobility. The area percentages were comparable to HPAEC, but in contrast to HPAEC the area percentage determined by CE-LIFD is equal to the molar percentage due to the fluorescent label on the reducing end. In the electropherogram from CASS the oligomer XLFG was the main oligomer (35 %), followed by XXFG (31 %) and XXXG (28 %). XLXG (2 %), XXLG (2 %), and XLLG (1 %) were minor oligomers. XLXG and XXLG were identified by their MS² spectra. Similar results were obtained using by HPAEC, but with RP-HPLC-ELSD analysis shows a higher area percentage of XLFG. Acetylated oligomers present in AIS digest were detected by CE-MS. Monoacetylated oligomers were not separated from the oligomers without an acetyl group, but XLFG carrying two acetyl groups was. Thus, a double peak was detected in the electropherogram of xyloglucan oligomers derived from AIS, where a single peak of XLFG was detected in the electropherogram of xyloglucan oligomers derived from CASS. With CE the different compositions of AIS xyloglucan compared to CASS xyloglucan was seen. In AIS xyloglucan XXFG (32 %) was the main xyloglucan oligosaccharides followed by XXXG (30 %) and XLFG (25 %). These results differed from the results of the HPAEC determination where XXXG was the main oligomer. The differences were, however, small and can be explained by different response factors on HPAEC-PAD (Hotchkiss and Hicks, 1990).

Xyloglucan oligomers of tamarind and cotton showed the same order of electrophoretic mobility under similar CE-LIFD conditions (Bauer et al., 2005). The authors do not give measurement times, but most likely our results are comparable due to similar systems used.

CE-LIFD is a time and solvent saving technique. Resolution of the oligomer separation is better and faster compared to HPAEC and RP-HPLC. The possibility to connect CE online to an ESIMS allows the identification of known and unknown xyloglucan oligomers and, therefore, their electrophoretic mobility. CE also separates double acetylated oligomers from single or non-acetylated ones. These advantages compensate the necessity to label the oligomers.
Figure 4.9 Electropherograms of APTS labelled xyloglucan oligosaccharides derived from black currant CASS (A) and AIS (B). In brackets the m/z values of the triple charged, labelled oligomers are shown. Acetylation is indicated by _ and maltose was used as an internal standard.

Figure 4.10 Electropherograms of APTS labelled xyloglucan oligosaccharides derived from black currant AIS detected by ESIMS.
Conclusions

We presented different techniques to analyse the structure of xyloglucan in black currants. All three separation techniques (HPAEC, RP-HPLC, and CE) show different elution orders of xyloglucan oligosaccharides. With each of these techniques it is possible to characterise xyloglucans by their oligomers obtained after enzyme degradation. HPAEC and CE showed similar separation profiles, while RP-HPLC was not able to separate all oligomers. MALDI-TOF MS and ESIMS can be used either instead, or additionally, or coupled off line to HPAEC or online to RP-HPLC or CE-MS, as newly presented in this paper. Combination of different techniques allows oligosaccharides profiling and structural characterisation of unknown oligosaccharides leading to an unambiguous xyloglucan structure. If it is necessary to limit the techniques, CE-LIFD is the fastest way to quantify xyloglucan oligomers. In case of unknown oligomers present, identification by online ESIMS is possible. MALDI-TOF MS can be used for fast oligosaccharide profiling, because many samples can be analysed in a short time. For structural characterisation ESIMS on a sensitive device outclasses PSD.

Black currant xyloglucan is of XXXG-type and contains galactose (L) and fucose-galactose (F) side chains in three major (XXXG, XXFG, and XLFG) and four minor building blocks (XXG, XLXG, XXLG, and XLLG). Black currant xyloglucans are the first xyloglucan analysed in the Grossulariaceae family and the Saxifragales order.

Acknowledgment

We thank Vernu Vasunta, Kiantama Ltd., Finland, for providing us with commercial berry material, Mirja Mokkilä and Kaisa Poutanen, VTT, Finland, for managing the project, Edwin Bakx and Jean-Paul Vincken, Laboratory of Food Chemistry, Wageningen University, The Netherlands, for building the CE-MS interface and the fruitful discussions about xyloglucan, respectively.

This study has been carried out with financial support from the Commission of the European Communities, specific RTD programme ‘Quality of Life and management of Living Resources’, contract number QLK1-CT-2002-02364 ‘Novel enzyme-aided extraction technologies for maximized yield and functionality of bioactive components in consumer products and ingredients from by-products’, acronym MAXFUN. It does not necessarily reflect its views and in no way anticipates the Commission’s future policy in this area.
Chapter 5

Bilberry xyloglucan – novel building blocks containing beta-xylose within a complex structure

Published as
Abstract

Bilberries have one of the most complex xyloglucan structures described in the plant kingdom until now. To characterise this structure, xyloglucans were enzymatically degraded and the oligosaccharides obtained were analysed. More than 20 different building blocks were found to make up the xyloglucan polymer. Bilberry xyloglucan was of XXXG-type, but some XXG-type oligomers were present, as well. The building blocks contain galactose-xylose (L) and fucose-galactose-xylose (F) side chains. In both side chains the galactose units can be acetylated. In addition, beta xylose-alpha-xylose (U) side chains were shown. This U chain was present in three building blocks described before (XUXG, XLUG, and XUFG) and four novel blocks that have not been described in literature previously: XUG, XUUG, XLUG, and XXUG.

Keywords
Vaccinium myrtillus; bilberry; cell wall polysaccharides; xyloglucan; hemicellulose; beta-xylose; Ericales; berries; capillary electrophoresis
Introduction

Xyloglucans are the main hemicelluloses in cell walls of dicotyledons (Vierhuis et al., 2001; Hoffman et al., 2005), where they were first described in sycamore cells (Bauer et al., 1973).

As an interlink between cellulose microfibrils, xyloglucans are assumed to be part of the skeletal framework as depicted in several models of the plant cell wall (Keegstra et al., 1973; Hayashi et al., 1987). Within the cellulose-xyloglucan network three xyloglucan domains are described: the first domain includes the parts of xyloglucans that bridge the space between cellulose microfibrils or that form free loops. This domain can be degraded by endo-glucanases. The major part of xyloglucans belongs to the second domain that covers the cellulose microfibrils and can be extracted with concentrated alkali. The third domain of the xyloglucans is entrapped within the amorphous cellulose microfibrils, which have to be degraded before the xyloglucan is accessible for enzymatic degradation or extraction (Hayashi et al., 1987; Pauly et al., 1999a; Bootten et al., 2004).

A $\beta$-(1→4)-linked glucan chain forms the backbone of xyloglucans (Aspinall and Molloy, 1969). Different short side chains are attached in position 6 to this backbone (Bauer et al., 1973). These chains were assigned a one-letter code to simplify nomenclature (Fry et al., 1993). Some examples for the side chains are G for an unsubstituted $\beta$-D-glcp, X for an $\alpha$-D-xylp(1→6)-$\beta$-D-glcp unit, L for a $\beta$-D-galp(1→2)-$\alpha$-D-xylp(1→6)-$\beta$-D-glcp unit, F for a $\alpha$-L-fucp(1→2)-$\beta$-D-galp(1→2)-$\alpha$-D-xylp(1→6)-$\beta$-D-glcp unit, or S for an $\alpha$-L-araf(1→2)-$\alpha$-D-xylp(1→6)-$\beta$-D-glcp unit.

Xyloglucans can be degraded by endo-glucanase in a regular way. Oligomers formed always contain an unbranched glucose on the reducing end. The polymeric xyloglucans are classified by the types of oligomers formed after enzymatic degradation. Xyloglucan oligomers of XXXG-type, XXGG-type, or XXGGG-type structure are the most common patterns (Buckeridge et al., 1997; Vincken et al., 1997). XXXX-type xyloglucan of which every glucosyl residue in the backbone is substituted with arabinosyl residues in position 2 was also reported (Mabusela et al., 1990).

The structure of xyloglucans is regular and similar within one botanic family (Hoffman et al., 2005). Bilberries belong to the family of Ericaceae, order Ericales. The xyloglucan structure of one other member of this order has been described before: the argan tree (Argania spinosa (L.) Skeels), which belongs to the family of Sapotaceae (Ray et al., 2004). In these xyloglucans a side chain of $\beta$-D-xylp(1→2)-$\alpha$-D-xylp(1→6)-$\beta$-D-glcp was identified for the first time and encoded with U. The building block XUFG was identified by NMR analysis. Based on MS data only, XUXG and XULG were proposed to be present, as well.

Bilberries (Vaccinium myrtillus L.) are an important crop in northern Europe due to their unique taste and flavour, although they are not domesticated. The berries are mainly used for juice production. We characterised cell wall polysaccharides of bilberries recently and showed high amounts of xylose and...
Structure of bilberry xyloglucans

glucose in their hemicellulose rich fraction, indicating the presence of xyloglucan (Hilz et al., 2005; Hilz et al., 2006b).

Aim of this study was to analyse xyloglucan from bilberries. To show the presence of xyloglucans and to characterise their structure, xyloglucans were degraded by xyloglucan specific endo-glucanase (XEG) (Pauly et al., 1999b) and the obtained oligomers were analysed using HPAEC, CE, and mass spectrometry.

Materials and Methods

Material

Bilberries (Vaccinium myrtillus L.) were obtained from Kiantama Ltd., Suomussalmi, Finland. Alcohol insoluble solids (AIS) were prepared from homogenised berries. In three extraction steps (hot buffer, chelating agent, 50 mM sodium hydroxide) the pectic polysaccharides were extracted from AIS. The hemicelluloses were extracted from the residual cellulose with 6 M sodium hydroxide (concentrated alkali soluble solids; CASS) as described previously (Hilz et al., 2005).

Fractionation of hemicelluloses with anion exchange chromatography

About 250 mg CASS were dissolved in water and the solution was centrifuged (10000 x g). The supernatant was applied on a DEAE Sepharose Fast Flow column (100 x 2.6 cm, GE Healthcare, Chalfont St. Giles, United Kingdom) and the unbound material was eluted with 530 mL 5 mM sodium acetate at pH 5.0 (CASS unbound) (Huisman et al., 2000).

Degradation of xyloglucan with xyloglucan specific endo-glucanase (XEG)

Xyloglucan containing material (5 mg) was dissolved in 1 mL 50 mM sodium acetate buffer (pH 5.0) and incubated with 1 µL xyloglucan specific endo-glucanase (XEG, EC 3.2.1.151 from Aspergillus aculeatus, 2259 U/mL) (Pauly et al., 1999b) over night. AIS (5 mg/mL) were incubated with additional 5 µL polygalacturonase (EC 3.2.1.15 from Kluyveromyces fragilis, 16 U/ml) (Daas et al., 1998) and 1 µL pectin methyl esterase (EC 3.1.1.11 from Aspergillus niger, 180 U/ml) (Baron et al., 1980) for pectin degradation (Vierhuis et al., 2001).

Sugar composition as alditol acetates

For determining the sugar composition, samples were pre-hydrolysed using 72 % w/w sulphuric acid at 30 °C for 1 h and subsequently hydrolyzed with 1 M sulphuric acid at 100 °C for 3 h (Saeman et al., 1945). Afterwards the sugars were derivatised to their alditol acetates and determined by gas chromatography (Englyst and Cummings, 1984) using inositol as internal standard.

Uronic acid content.

The total uronic acid content was determined photometrically with the automated m-hydroxydiphenyl assay (Thibault, 1979).
Chapter 5

**HPAEC of xyloglucan oligomers**

Xyloglucan oligosaccharides were analysed on a CarboPac PA 100 column (4 x 250 mm, Dionex, Sunnyvale, USA) and on a CarboPac PA 1 column (2 x 250 mm, Dionex, Sunnyvale, USA) with pulse amperometric detection (PAD) using a column specific sodium hydroxide/sodium acetate-gradient as described previously (Vincken et al., 1996) and a flow of 1 mL/min for the 4 x 250 mm column and of 0.3 mL/min for the 2 x 250 mm column, respectively.

For desalting a FC-203B fraction collector (Gilson, Middleton, USA) was connected that collected fractions of 30 s directly after the PAD. These fractions were collected in a Sep-Pak tC18 (40 mg) 96-well plate (Waters, Milford, USA) containing 0.5 mL 0.5 M acetic acid per well. The eluent was sucked through the Sep-Pak tC18 (40 mg) 96-well plate by vacuum with a MultiScreen resist vacuum manifold (Millipore, Billerica, USA). After washing two times with 1 mL water, the oligomers were eluted with 2 mL methanol and the fractions were dried at 60 °C (Hilz et al., 2006a).

Preparative HPAEC was performed on an preparative CarboPac PA 100 column (22 x 250 mm, Dionex, Sunnyvale, USA). The same gradient as on analytical scale was applied with a flow of 18 mL/min. Fractions were collected every 30 s.

**MALDI-TOF MS**

Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) was performed using an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm and operated in positive mode. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12000 V and detected using reflector mode. The lowest laser power required to obtain good spectra was used and at least 100 spectra were collected. The mass spectrometer was calibrated with a mixture of maltodextrins (mass range 350-2350).

The xyloglucan oligomers were desalted using Sep-Pak tC18 (20 mg) cartridges (Waters, Milford, USA) (Hilz et al., 2006a). 2 µL sample solution were mixed on the MALDI-TOF-plate (Bruker Daltonics, Bremen, Germany) with 2 µL matrix solution of 9 mg/mL 2,5 dihydroxybenzoic acid (Bruker Daltonics, Bremen, Germany) in 30 % acetonitrile and dried under a stream of air (Verhoef et al., 2005).

**ESIMS**

Electrospray ionisation mass spectrometry (ESIMS) was performed on a LTQ Ion-trap (Thermo Electron, San Jose, USA). The sample was applied through a PicoTip emitter capillary (4 µm ID of the tip, New Objective, Woburn, USA). MS analysis was carried out in the positive mode using a spray voltage of 1.5 kV and a capillary temperature of 200 °C. The instrument was auto-tuned on xyloglucan oligosaccharides. MS² and higher was performed using a window of 1 m/z and a relative collision energy of 30 % (Hilz et al., 2006a).
CE-LIFD

For capillary electrophoresis (CE) xyloglucan oligosaccharides we labelled with 8-aminopyrene-1,3,6-trisulfonate (APTS) using the ProteomeLab Protein Characterisation kit (Beckman Coulter, Fullerton, USA).

The labelled oligosaccharides were separated on a polyvinyl alcohol (N CHO) coated capillary (50 µm ID x 50.2 cm, detector after 40 cm, Beckman Coulter, Fullerton, USA) using a ProteomeLab PA 800 characterisation system (Beckman Coulter, Fullerton, USA) and a laser induced fluorescence detector (LIFD) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm (Beckman Coulter, Fullerton, USA). The separation was carried out in reversed polarity at 30 kV in a 25 mM acetate buffer containing 0.4 % polyethylene oxide at pH 4.75. The capillary was kept at 25 °C (Hilz et al., 2006a).

CE-ESIMS

For identification of the different oligomers, separation was carried out on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) with a fused silica capillary (50 µm ID x 80 cm) connected to a UV-detector (after 20 cm) and an ESIMS<sup>n</sup> detector (LTQ Ion-trap, Thermo Electron, San Jose, USA) by a device made in our laboratory. The separation was carried out in reversed polarity at 28 kV in a 50 mM ammonium acetate buffer containing 0.2 % formic acid at pH 2.8. The capillary was kept at 15 °C. For ESI MS<sub>n</sub> a sheet flow of 4 µL/min of 75 % isopropanol in water was used. ESIMS<sub>n</sub> was operated in the negative mode using a spray voltage of 2.2 kV and a capillary temperature of 190 °C. The instrument was auto-tuned on xyloglucan oligosaccharides. MS<sub>2</sub> and higher was performed using a window of 1 m/z and a relative collision energy of 30 % (Hilz et al., 2006a).

<sup>1</sup>H NMR

Prior to NMR analyses, the samples were reduced with sodium borohydrate (York et al., 1990), desalted using Sep-Pak tC18 (20 mg) cartridges (Waters, Milford, USA) and the reduced xyloglucan oligomers were dissolved in 99.96 % D<sub>2</sub>O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved in 99.996 % D<sub>2</sub>O (Cambridge Isotope Laboratories, USA). <sup>1</sup>H NMR spectra were recorded at a probe temperature of 300 K on a AMX-500 spectrometer (Bruker BioSpin, Rheinstetten, Germany) located at the Wageningen NMR Centre. Chemical shifts were expressed in ppm relative to internal acetone: 2.225 ppm. The 1D <sup>1</sup>H proton spectra were recorded with pre saturation of the HOD signal (70 dB) at 500.13 MHz using 256 scans of 8192 data points and a sweep width of 6000 Hz.

Results

Xyloglucans of bilberries were analysed following two approaches. In a first approach, hemicelluloses were extracted with 6 M sodium hydroxide from residual cell wall material after removal of pectins (concentrated alkali soluble solids; CASS). Xyloglucans were further purified on an anion exchange column,
where neutral hemicelluloses including xyloglucan were separated from anionic hemicelluloses and remaining pectins. Under these alkali extraction conditions possible acetyl esters were saponified and could not be determined. Therefore, xyloglucans were degraded with XEG directly starting from alcohol insoluble solids (AIS) in a second approach. During this procedure possible acetyl esters remained intact on the obtained oligosaccharides.

**Sugar composition of xyloglucan containing fractions**

For a first characterisation of polysaccharides in CASS and AIS, the sugar composition after hydrolysis of the polysaccharides was determined (table 5.1). In CASS, xylose and glucose were the major sugar residues with a ratio of xylose to glucose typical for xyloglucan (3:4). Only small amounts of uronic acids were present in this fraction. These uronic acids were five time more galacturonic acids than glucuronic acids (Hilz et al., 2005).

Purer xyloglucan was obtained when CASS was further fractionated on an anion exchange column (Hilz et al., 2006a). Eighteen percent of CASS did not bind to the column and eluted with 5 mM sodium acetate buffer (CASS unbound). This fraction contained partly purified xyloglucan as indicated by its sugar composition (table 5.1). CASS unbound contained xylose and glucose in a ratio of 2:4. The amount of fucose was relatively high in this fraction and arabinose was hardly present. The amount of uronic acids was low. Probably xyloglucans with fucose and galactose containing side chains (galactose content 13 mol %) were present and arabinose was not part of bilberry xyloglucans.

The major sugar residues in bilberry AIS were xylose, glucose, and uronic acid. The molar ratio of xylose to glucose was almost 1:1 showing that not only xyloglucan, but also xylans were present in the cell wall material of bilberries. Bilberry seeds contain high amounts of xylans (Hilz et al., 2005).

**Table 5.1 Sugar composition of xyloglucan containing fractions of bilberries [mol %].**

<table>
<thead>
<tr>
<th>Rha</th>
<th>Fuc</th>
<th>Ara</th>
<th>Xyl</th>
<th>Man</th>
<th>Gal</th>
<th>Glc</th>
<th>UA</th>
<th>Total sugars [w/w %]</th>
<th>Yield</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td>CASS</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>28</td>
<td>11</td>
<td>12</td>
<td>38</td>
<td>6</td>
<td>4 % of AIS</td>
</tr>
<tr>
<td>CASS unbound</td>
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<td>4</td>
<td>1</td>
<td>19</td>
<td>16</td>
<td>13</td>
<td>42</td>
<td>6</td>
<td>18 % of CASS</td>
</tr>
<tr>
<td>AIS</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>29</td>
<td>2</td>
<td>5</td>
<td>35</td>
<td>22</td>
<td>40 % of fresh berries</td>
</tr>
</tbody>
</table>

**Profiling of xyloglucan oligomers by MALDI-TOF MS and ESIMS**

Endoglucanase II is a xyloglucan specific endo-glucanase (XEG), which is known to split xyloglucan between an unbranched glucose and a xylose-substituted glucose residue (Pauly et al., 1999b). By incubation with XEG, xyloglucans are degraded into specific oligosaccharides representing the building blocks of xyloglucans (Fry et al., 1993), which can have XXXG type, XXGG type, or the XXGGG type structure (Buckeridge et al., 1997; Vincken et al., 1997).
Figure 5.1A MALDI-TOF mass spectrum of xyloglucan oligosaccharides derived from bilberry CASS (m/z of sodium adducts between brackets). The side chain (1H+2P) was later identified as the U chain by NMR.

Figure 5.1B MALDI-TOF mass spectrum of xyloglucan oligosaccharides derived from bilberry AIS (m/z of sodium adducts between brackets). The side chain (1H+2P) was later identified as the U chain by NMR.
MALDI-TOF MS of XEG digested xyloglucans indicates the presence of different oligomers by their mass to charge ratios. When ESIMS\textsuperscript{n} is performed, the structure of the oligomers can be identified. However, the different hexoses and pentoses cannot be distinguished. By comparing xyloglucan oligomers obtained from CASS with the ones obtained from AIS, acetylation profiles can be seen (Vierhuis et al., 2001; Hoffman et al., 2005).

The MALDI-TOF mass spectrum of xyloglucan oligomers derived from bilberry CASS is shown in figure 5.1A. In this spectrum 19 peaks were present. 15 peaks were attributed to sodium adducts of xyloglucan oligosaccharides and four peaks were attributed to potassium adducts (1233, 1263, 1425, 1571), which have a mass to charge ratio of 16 higher than their corresponding sodium adducts. The xyloglucan oligomers XXXG (1085), XXLG and XLXG (X[XL]G; 1247) XXFG (1393), XLLG (1409), and XLFG (1555) were identified according to their mass to charge ratio (Hoffman et al., 2005) and their ESIMS\textsuperscript{2} spectra. Other major peaks were present, which were attributed to oligomers that contained either a side chain of two pentoses or two pentoses attached to one glucose residue of the backbone. Until further identification this side chain is coded as (1H+2P). The main xyloglucan oligomers containing this side chains were X[X(1H+2P)]G (1217), X(1H+2P)(1H+2P)G (1349), X[L((1H+2P)]G (1379), and X(1H+2P)FG (1525). Some smaller peaks present were attributed to X(1H+2P)G (923), XLG (953), and XLGG (1115). The ESIMS\textsuperscript{2} spectrum of the peak with the mass to charge ratio of 1085 suggested the presence of small amounts of X(1H+2P)GG next to XXXG. Three oligomers with the mass to charge ratios of 1277, 1511, and 1541, respectively, were shown to carry a substituted xylose residue at the non-reducing end. We propose L[LG]G, L(1H+2P)(1H+2P)G and L(1H+2P)LG as most probable structures based on ESIMS\textsuperscript{n} analysis, because \textsuperscript{1}H NMR analysis did not result in conclusive spectra (data not shown). This would mean that XEG is, under special circumstances, able to release xyloglucan oligosaccharides with an L-unit on the non-reducing end.

Identification of the sugar residues carrying the acetyl group by ESIMS\textsuperscript{n}

The MALDI-TOF mass spectrum of xyloglucan oligosaccharides derived from AIS showed many more peaks (figure 5.1B) than the one of enzymatically degraded CASS xyloglucans. The additional peaks were the single or double acetylated oligomers. A peak with the mass to charge ratio of XXG (791) was shown additionally compared to the spectrum of xyloglucan oligosaccharides derived from CASS. Two other additional peaks had the mass to charge ratio of 1055 and 1097, respectively. These peaks were attributed to an oligomer composed of 4 pentoses and 3 hexoses in the acetyl free and the single acetylated form. Its structure, however, could not be identified. Two peaks were not attributed to xyloglucan oligosaccharides (1229, 1481).

By ESIMS\textsuperscript{n} the sugar residues that carry the acetyl group were identified. Only oligomers containing a galactose unit in an L or F chain and oligomers containing another G unit next to the reducing end were shown to be acetylated. ESIMS\textsuperscript{n} analysis confirmed acetylation of the galactose units and the
unsubstituted, non-reducing glucose units. When double acetylation was shown on one molecule, no double acetylated residues, but two different acetylated galactose residues or an acetylated galactose and an acetylated glucose unit were present. Among the acetylated xyloglucan oligomers, one peak with a mass to charge ratio of 1127 (1085 + acetyl) was detected. This oligomer was attributed to the tentative structure of acetylated X(1H+2P)GG. The L[LG]G and L[(1H+2P)L]G oligomers were shown in the MALDI-TOF mass spectrum of xyloglucan derived from AIS, as well, L[LG]G even in the single and double acetylated form.

**Xyloglucan oligosaccharides of bilberries analysed by HPAEC**

The HPAEC elution pattern (CarboPac PA 100 column) of xyloglucan oligomers derived from bilberry CASS unbound is shown in figure 5.2. The high number of different oligomers made it difficult to separate all oligomers. Bilberry xyloglucans consisted of more than 20 different oligomeric building blocks. For identification retention times of known xyloglucan oligosaccharides from soy (Huisman et al., 2000), olive (Vierhuis et al., 2001), apple (Renard et al., 1992), and tamarind (Vincken et al., 1995) were used. Because not all xyloglucan oligomers were identified by this way, off line MALDI-TOF MS and ESIMS were performed after desalting by solid phase extraction (Hilz et al., 2006a) in order to identify the still unknown peaks and to confirm the structure of the identified ones.

The smaller oligomers XXG, XFG, and XLG eluted first from the HPAEC column. These oligomers with a backbone of three glucose units were identified by their mass to charge ratios (MALDI-TOF MS) and their ESIMS spectra. XFG was not detected by MALDI-TOF MS of the xyloglucan oligomer mixtures derived from CASS or AIS. These XXG-type oligomers were present as building blocks of bilberry xyloglucans in minor quantities. The major building blocks were of XXXG-type. The first xyloglucan oligomer of XXXG-type eluting from the column was XXXG followed by XXFG, XLFG, and XXL G. XLLG was present in smaller amounts. More oligomers carrying the (1H+2P) side chain were identified by their mass to charge ratios (MALDI-TOF MS) and their ESIMS spectra.

**Identification of the xyloglucan oligosaccharides by HPAEC-off line-ESIMS**

The ESIMS spectra of the xyloglucan oligomers separated by HPAEC were spectra of single oligomers. Even if two oligomers were coeluting, they did not have the same molecular mass. The ESIMS spectrum of XX(1H+2P)G 1 is shown in figure 5.3 as an example for ESIMS analysis. The peaks present were assigned to fragments of the molecule according to Domon and Costello (1988). Three peaks in the spectrum gave important information about the structure of the oligomer. One of them was the peak with the mass to charge ratio of the four glucose units in the backbone (689). This ion was formed by cleavage of four non-reducing pentose residues resulting in the Y-ion of the backbone. The other peaks represented the fragments which were formed by cleavage in the middle of the
Figure 5.2 Oligomers obtained by incubation of isolated bilberry xyloglucan (CASS unbound) with XEG on HPAEC CarboPac PA100 column. The side chain (1H+2P) was later identified as the U chain by NMR.

Figure 5.3 ESIMS² spectrum of XX(1H+2P)G (structure in the upper left corner. (1H+2P) was later identified as U by NMR).
Structure of bilberry xyloglucans

oligomer. The peak with a mass to charge ratio of 611 could be either the B3α' or the Z2α' ion. Probably a mixture of the ions was present. The peak with a mass to charge ratio of 629 was probably a mixture of the corresponding ion C3α' and Y2α', respectively. These two peaks showed that two xylose units were attached to the glucose unit of the chain next to the reducing end (a). The other oligomers that contained the (1H+2P) side chain were identified as XL(1H+2P)G, X(1H+2P)LG, X(1H+2P)FG, and X(1H+2P)(1H+2P)G. Three oligomers with the same masses (m/z (M+Na+) = 1217) and identical ESIMS spectra were eluted three different retention times for the HPAEC conditions used. They carried the (1H+2P) side chain next to the reducing end and were labelled XX(1H+2P)G 1-3, although the reason for the different physico-chemical behaviour of the three oligomers on HPAEC cannot be explained. A similar phenomenon was observed for the oligomers carrying a (1H+2P) and an L side chain (m/z (M+Na+) = 1379). Next to XL(1H+2P)G and X(1H+2P)LG a third oligomer eluted from the column at a different retention time. The ESIMS spectrum did not give conclusive spectra. Therefore, the oligomer was labelled X[L(1H+2P)]G. In very small quantities, two oligomers with a mass to charge ratio two higher than XX(1H+2P)G or XXLG were shown. According to their ESIMS spectra, they could be the reduced forms XX(1H+2P)Gol and XXLGol, which may be artefacts of the procedure or originally present on the reducing end of xyloglucan.

CE of bilberry xyloglucan oligosaccharides

In addition to HPAEC, capillary electrophoresis (CE) was used to separate especially acetylated xyloglucan oligosaccharides (Bauer et al., 2005; Hilz et al., 2006a). Xyloglucan oligosaccharides derived from bilberry CASS (figure 5.4A) and AIS (figure 5.4B) were labelled with APTS on the reducing end and the electropherograms were recorded using a laser induced fluorescence detector. Most of the many different oligomers in bilberry xyloglucan were separated within only two minutes. Since LIFD is not able to identify oligomers, CE was also connected online to ESIMS. Next to the letter codes, the mass to charge ratios of the triple negatively charged, APTS labelled oligomers are shown in the electropherograms.

The electrophoretic mobility of the APTS-labelled oligosaccharides derived from CASS depended on the molecular mass of the oligomers. The oligomers with the lowest molecular mass migrated the fastest to the detector and the ones with the highest molecular mass the slowest. In the beginning of the electropherogram the resolution was better compared to the end. While XXLG and XLLG with the same mass were separated in different peaks, XL(1H+2P)G, X(1H+2P)LG, and XXFG were detected in one peak. The same major oligomers were detected by CE-LIFD as by HPAEC. It was, however, not possible to identify the oligomers present in the CASS digest in smaller amounts by their masses due to the short separation time and therefore the limited number of mass spectra.

When acetylated oligomers derived from bilberry AIS were analysed, the acetyl free and the monoacetylated forms were not separated from each other. The
Figure 5.4A CE-LIFD electropherograms of xyloglucan oligomers derived from bilberry CASS. Oligomers were identified by CE-MS according to their triple charged APTS oligomers (m/z between brackets). The side chain (1H+2P) was later identified as the U chain by NMR.

Figure 5.4B CE-LIFD electropherograms of xyloglucan oligomers derived from bilberry AIS. Oligomers were identified by CE-MS according to their triple charged APTS oligomers (m/z between brackets). The side chain (1H+2P) was later identified as the U chain by NMR.
Structure of bilberry xyloglucans

diacetylated oligomers were, however, separated from the acetyl free and the monoacetylated oligomers, for example in the case of XLFG and XLLG. In addition, higher amounts of the smaller oligomers were present, so that an identification of the oligomers with an electrophoretic mobility between XXG and XXXG was partly possible. However, some peaks remained unidentified. An oligomer with the mass of three hexoses and four pentoses, which was already shown to be present by MALDI-TOF MS, was attributed to well separated peak. This oligomer was not detected by HPAEC.

Identification of novel xyloglucan oligosaccharides by \textsuperscript{1}H NMR

To identify the structure of oligomers containing the unknown (1H+2P) chain, four oligomers (XX(1H+2P)G, X(1H+2P)FG, XL(1H+2P)G, and X(1H+2P)(1H+2P)G) were isolated by preparative HPAEC (Vierhuis et al., 2001). The reducing ends of the oligomers were reduced to alditols prior to \textsuperscript{1}H NMR. The chemical shifts and coupling constants (table 5.2) were compared to previously recorded spectra of xyloglucan oligosaccharides with (Ray et al., 2004) and without (York et al., 1990) the \(\beta\)-xylose containing side chain (U) or the arabinose containing side chain (S) (Vierhuis et al., 2001). The position of the sugar residues in the oligomers were indicated by a letter (see structure in figure 5.3) that was used before for xyloglucan oligosaccharides (York et al., 1990), starting with a c for the side chain on the non-reducing end.

Table 5.2 \textsuperscript{1}H NMR chemical shifts and coupling constants for reduced xyloglucan oligomers of bilberries, tentatively assigned according to literature. The positions of the different sugar residues are labelled by letters as described before (York et al., 1990; Ray et al., 2004).

<table>
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* may be interchanged

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The spectrum of XX(1H+2P)G showed the anomeric proton doublet of the β-D-xylose (δ = 4.58, J1,2 = 7.37) forming the U-side chain. Furthermore, two unsubstituted α-D-xylose (δ = 4.94, J1,2 = 3.53 and δ = 4.96, J1,2 = 3.58) and one substituted α-D-xylose (δ = 5.12, J1,2 = 3.66) were shown to be present. In the anomeric region proton doublets of the other sugar residues were shown. The (1H+2P) side chain was identified as the U-side chain, which was previously reported in xyloglucans form the argan tree (Ray et al., 2004). In X(1H+2P)(1H+2P)G two anomeric proton doublets of β-D-xylose (δ = 4.57, J1,2 = 7.35 and δ = 4.57, J1,2 = 8.04) were seen. Each of them was probably linked to an α-D-xylose (δ = 5.12, J1,2 = 3.60 and δ = 5.15, J1,2 = 3.17), leaving the α-D-xylose of the non-reducing end free (δ = 4.95, J1,2 = 3.16). This oligomer contained two U-side chains and was identified as XUUG. XL(1H+2P)G contained a β-D-xylose unit (δ = 4.57, J1,2 = 7.89), as well. The β-D-galactose unit could be identified (δ = 4.56, J1,2 = 7.56) and the β-D-galactose and the β-D-xylose were attributed to two substituted α-D-xyloses (δ = 5.14, J1,2 = 3.15 and δ = 5.163, J1,2 = 2.75). This oligomer was identified as XLUG. X(1H+2P)FG showed the typical α-L-fucose residue (δ = 5.27, J1,2 = 3.70) and the substituted β-D-galactose (δ = 4.61, J1,2 = 6.93) next to the β-D-xylose unit (δ = 4.57, J1,2 = 7.40). The oligomer was identified to be XUFG.

**Contribution of oligomeric building blocks to xyloglucan structure**

To compare the composition of XEG digests from CASS and AIS, the peak area% of the different oligomers after separation by HPAEC (CarboPac PA 1 and CarboPac PA 100) and CE were calculated (table 5.3). With HPAEC-PAD the area depends not only on the concentration, but on the response factors of different oligosaccharides (Hotchkiss and Hicks, 1990), as well. However, no big differences in response factors between the oligomers were expected, because the xyloglucan oligomers were of similar structure and size. In the HPAE-chromatogram of the CASS digest XXUG was the main oligomer followed by XXFG, XXXG (probably coeluting with an oligomer tentatively assigned as XUGG), XXLG, and XLFG. These oligomers were present to more than 10 %. Comparing the elution patterns of digest separated on the two different columns, XXLG contributed with 11 %, XLFG with 10 %, and XLXG with less than 1 % to the total area of xyloglucan oligosaccharides. While XXUG 2 and XLUG coeluted on CarboPac PA 100, separation of CarboPac PA 1 showed the presence of these two oligomers in similar amounts. Similar to black currants (Hilz et al., 2006a), the building block composition in CASS was the same as in CASS unbound and the not further described fractions from DEAE. When comparing the xyloglucan oligomers present in CASS with the oligomers present in AIS, different profiles of the xyloglucan oligomers were observed. From AIS more XG-type oligosaccharides were released. Furthermore, more XXXG (probably coeluting with an oligomer tentatively assigned as XUGG) was present in the AIS digest. Less XXUG 1 and XUGF were determined instead. XXLG was the main oligosaccharide present in the XEG digest of bilberry AIS.
Using CE-LIFD rather similar results as by HPAEC-PAD were observed. Variations of the two methods were due to different response factors of HPAEC. In CE-LIFD the area% composition is equal to the mole% composition due to labelling of the reducing end. Only the amount of XXUG in xyloglucan of CASS was present in a lower percentage when determined by CE-LIFD compared to HPAEC-PAD.

Table 5.3 Quantification of oligomers derived from xyloglucans of bilberries as analysed on HPAEC PA100, HPAEC PA1 and CE. The oligomers were identified by their masses, off line by MALDI-TOF MS and ESIMS\(^a\) for HPAEC and online with ESIMS\(^e\) for CE [Area %].

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<th>M + APTS(^b)</th>
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<th>HPAEC PA1</th>
<th>CE</th>
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<td>10 5</td>
</tr>
</tbody>
</table>

\(^a\) coelution with XUGG; \(^b\) coelution of all X[XU]G oligomers; \(^c\) coelution with XLUG; \(^d\) coelution with all X[UL]G; \(^e\) coelution with X[LU]G; \(^f\) coelution with XULG and XXUG 3; \(^g\) coelution with XULG; \(^h\) coelution with XXLG

**Discussion**

Bilberry xyloglucans were analysed after degradation of xyloglucans with XEG. Compared to the known xyloglucan structures (Ray et al., 2004; Hoffman et al., 2005; Hilz et al., 2006a), bilberry xyloglucans were more complex. HPAEC and CE were again shown to be powerful tools for the characterisation of xyloglucan oligosaccharides (Hilz et al., 2006a), even when many oligosaccharides are present with similar structures. Both analytical techniques provided similar
results according to the composition of xyloglucans and used together give additional information about the structure.

The sugar composition of the isolated xyloglucans (CASS unbound) showed that xyloglucans contained galactose and fucose next to xylose and glucose. Arabinose was not present. However, MALDI-TOF MS, ESIMS<sup>n</sup>, and HPAEC coupled off line to MALDI-TOF MS or ESIMS<sup>n</sup> showed clearly the presence of xyloglucan oligomers carrying two pentoses on one glucose residue of the backbone. That these oligomers did not contain an arabinose containing side chain (S) was confirmed by HPAEC, when xyloglucan oligomers derived from olives (Vierhuis et al., 2001) were analysed under the same conditions. The retention times of the S-side chain containing oligosaccharides were much higher than the retention times of the xyloglucan oligomers in the bilberries. Further indications for the structure of the unknown side chain were found in literature, where a regular and similar structure of xyloglucans is described for taxonomically related species (Vierhuis et al., 2001; Hoffman et al., 2005). Bilberries belong to the family of Ericaceae, order Ericales. The xyloglucan structure of one other member of Ericales has been described before: the argan tree (*Argania spinosa* (L.) Skeels), which belongs to the family of Sapotaceae (Ray et al., 2004). In these xyloglucans the XUFG building block was identified and XUXG and XULG were proposed for the corresponding masses.

By ESIMS<sup>2</sup> analysis, we were able to locate the position of the side chain consisting of two pentoses connected to one hexose. If the pentoses were connected to each other or both to the hexose had to be determined by <sup>1</sup>H NMR analysis of four isolated oligomers. All four contained a β-D-xylose attached to an α-D-xylose (the U side chain). In bilberry xyloglucans XUG, XXUG, XULG, XLUG, XUFG, and XUUG were identified. XUG, XUUG, XXUG, and XLUG are novel xyloglucan building blocks not described before. All four oligomers contained a β-D-xylose residue, which was unsubstituted and attached to an α-D-xylose residue.

Furthermore, acetylation of xyloglucan oligosaccharides obtained from AIS was shown. The acetyl groups are present on the galactose units of xyloglucan, as already shown in xyloglucan from sycamore cells (Kiefer et al., 1989). Major differences in the composition of xyloglucans present in CASS and degradable in AIS were seen. AIS contained more XXG-type oligosaccharides, which are known to be present in these xyloglucan domains (Pauly et al., 1999a). These differences are due to the different accessibility of xyloglucan domains (Hilz et al., 2006a). In AIS mainly xyloglucans that cross-link cellulososes or that are present in free loops are degraded, while in CASS xyloglucans that are hydrogen bound to cellulose are extracted, as well (Pauly et al., 1999a). Perhaps the acetylation of the galactose side chain may also have an effect on the degradation of xyloglucan in AIS (Hilz et al., 2006a), which would result in the observed differences in oligosaccharide composition.

In bilberry xyloglucans, small amounts of reduced oligomers (XXUGol and XXXLGol) were shown to be present. They may be artefacts of the isolation procedure. However, they were present in xyloglucan from AIS, where the
isolation procedure was mild and should not lead to chemical modification of oligomers. If these oligosaccharide-alditols were present in native xyloglucan, they can only be present on the reducing end.

Conclusions

Bilberries contain xyloglucans of XXXG-type structure, although different XXG-type oligomers are released in small amounts from AIS. XXG-type oligomers are known to be present in xyloglucan domains that are less tightly bond to cellulose (Levy et al., 1991; Pauly et al., 1999a). Next to the galactose (L) and the fucose-galactose (F) containing side chain, the β-D-xylose containing side chain (U) was identified. The galactose residues were partly acetylated. Bilberry xyloglucans were shown to have one of the most complex structures described in literature until now. They consist of more than 20 different building blocks, among them 4 novel ones. The presence of the U-side chain in 7 xyloglucan oligomers from bilberries was proven, showing that the U-side chain is a common element of xyloglucans in the order Ericales.

Acknowledgment

We thank Vernu Vasunta, Kiantama Ltd., Finland, for providing us with commercial berry material, Mirja Mokkila and Kaisa Poutanen, VTT, Finland, for managing the project, and Jean-Paul Vincken and Edwin Bakx, Laboratory of Food Chemistry, Wageningen University, The Netherlands, for the fruitful discussions about xyloglucan and mass spectrometry, respectively.

This study has been carried out with financial support from the Commission of the European Communities, specific RTD programme ‘Quality of Life and management of Living Resources’, contract number QLK1-CT-2002-02364 ‘Novel enzyme-aided extraction technologies for maximized yield and functionality of bioactive components in consumer products and ingredients from by-products’, acronym MAXFUN. It does not necessarily reflect its views and in no way anticipates the Commission’s future policy in this area.
Chapter 6

Combined enzymatic and high-pressure processing affect cell wall polysaccharides in berries

Published as
Abstract

The effect of high-pressure processing (HPP) on cell wall polysaccharides in berries was investigated. HPP decreased the degree of methyl esterification (DM), probably by activation of pectin methyl esterase (PME), and improved the extractability of pectins. When commercial enzyme mixtures were added to mashed berries, a synergistic effect was observed between treatment with commercial enzymes and HPP. Compared to treatment at atmospheric pressure, pectic polysaccharides were degraded to a larger extent when HPP was used. In contrast, hemicelluloses were hardly affected by the added enzymes when HPP was included, although they were degraded during similar treatment at atmospheric pressure. Additionally, the activity of rhamnose-releasing enzymes present in minor quantities might be enhanced after HPP, resulting in a decrease of rhamnose in the polymeric cell wall material. These results exploring the effect of HPP at representative conditions clearly point out the potential of HPP for polysaccharide modification.

Keywords
Vaccinium myrtillus; bilberry; Ribes nigrum; black currant; cell wall polysaccharides; fractionation; berries; high pressure; enzyme mixture; enzymes; cell wall
Introduction

The first experiments with high-pressure processing (HPP) on food were carried out with the purpose of enhancing the microbial stability of food products such as milk, meat, and fruit juices without changing the sensorial properties (Hite, 1899). New interest in this field rose at the end of the 1980s with the development of high pressure devices suitable for broad industrial food applications (Farr, 1990). Today, HPP is investigated with the aim of preserving foods, changing food texture, preserving flavor, or influencing enzymatic reactions (Farr, 1990; Hendrickx et al., 1998). Some HPP products are already on the market (Watanabe et al., 1991; Pfister and Dehne, 2001; Knorr, 2002; Ruiz-Capillas and Jimenez-Colmenero, 2004; Tabilo-Munizaga and Barbossa-Canovas, 2004). Fruit juices, fruits, jams, or other fruit products can be treated with high pressure for pasteurization or high pressure freezing (Watanabe et al., 1991; Pfister and Dehne, 2001). However, HPP remains an expensive treatment.

High pressure encourages those chemical reactions and structural rearrangements that lead to a decrease of the total volume, for example by changing the tertiary structure of proteins. Covalent bonds in proteins are hardly affected by high pressure, but ionic and hydrogen bonds and with them the tertiary structure can change drastically (Balny and Masson, 1993; Hendrickx et al., 1998). HPP-induced gelling was reported for proteins and amidated, low methyl esterified pectins (Farr, 1990; Abbasi and Dickinson, 2002).

To date, the effect of HPP on cell wall polysaccharides is unclear. The plant cell wall is composed of three main groups of polysaccharides: cellulose, hemicellulose, and pectins (McCann and Roberts, 1991). Cellulose forms together with hemicelluloses the firm backbone of the wall, which is embedded in a pectic network. This pectic network mainly influences the firmness of tissue and is responsible for textural changes during food processing, although the mechanism is not always clear.

How HPP affects endogenous pectinolytic enzymes was studied for polygalacturonase (PG) and pectin methyl esterase (PME) isolated from different fruits and vegetables (Ogawa et al., 1990; Seyderhelm et al., 1996; Ludikhuyze et al., 2003; Balogh et al., 2004; Fachin et al., 2004; Peeters et al., 2004; Verlent et al., 2004). All investigations show a fast decrease of PG activity with increasing pressure, but PME activity appears to be pressure stable or is even activated. How other cell wall degrading enzymes, for example of fungal origin or hemicellulases, behave under high pressure is not known.

Bilberries (Vaccinium myrtillus L.) and black currants (Ribes nigrum L.) are important crops in northern Europe. In the food industry most bilberries and black currants are processed to juice. This juice can be consumed as such or used as a food ingredient. During juice production the cell walls are ruptured and pectic polysaccharides form a strong pectin gel, from which juice can be obtained only with difficulty (Grassin and Fauquembergue, 1996). For better juice yields (Grassin and Fauquembergue, 1996) and improved extraction of phenolic
Effect of combined enzymatic and high pressure processing on cell wall polysaccharides compounds (Landbo and Meyer, 2001; Meyer, 2002; Bagger-Jorgensen and Meyer, 2004; Buchert et al., 2005) it has become common practice to use commercial enzyme mixtures, usually of fungal origin, which contain a variety of cell wall degrading enzymes, to degrade the pectin gel. In a recent publication we described the detailed composition of cell wall polysaccharides from bilberries and black currants (Hilz et al., 2005).

The research described in this study is part of a bigger research project aiming at maximizing the yield and functionality of berry products by using novel processing technologies such as high pressure or high-power ultrasound. Preliminary observations using HPP and commercial enzyme mixtures showed a clear effect on viscosity and extractability of bioactive compounds. The aim of this study was to investigate how enzymes affect cell wall polysaccharides in food when treated under high pressure conditions. This paper describes these effects observed for representative HPP conditions to better understand the potential of HPP to modify cell wall polysaccharides. We investigated changes in different classes of cell wall polysaccharides caused by HPP alone and in combination with commercial enzyme mixtures.

**Materials and methods**

**Material**

Commercial bilberries (V. myrtillus L.) were obtained from Kiantama Ltd., Suomussalmi, Finland. Black currants (R. nigrum L., cv. Öjeby) were obtained from Peltohermanni Ltd., Ilomantsi, Finland. Both berries were of the 2002 harvest and were stored frozen at -23 °C. Three commercial enzyme mixtures were used: (1) Biopectinase CCM, pectinolytic mixture (Kerry Biosciences, Tralee, Ireland); (2) Pectinex Ultra SP L, pectinolytic enzyme (Novozymes, Bagsvaerd, Denmark); and (3) Econase CE, hemicellulolytic enzyme (AB Enzymes, Darmstadt, Germany). Specific activities of glycanases present in the enzyme mixtures have been described before (Buchert et al., 2005).

**High-Pressure Processing**

Frozen bilberries or black currants (200 g) were spread onto a dish and allowed to thaw at 21 °C for 20 or 35 min, respectively. The partly thawed berries were mashed for 8 s (bilberries) or 40 + 5 s (black currants) in a kitchen mixer. For HPP, 20 g of the mash was packed into six small plastic pouches. The rest of the puree was left at room temperature during the pressure treatment and used as control sample in the analysis described below.

HPP was performed on a laboratory-scale, multivessel high-pressure device (HPIU-10000-AT, Resato International, Roden, The Netherlands). One sample pouch was placed into each of the six pressure vessels. The vessels were filled with pressure-transmitting medium and closed. The samples were pressurized to 400 MPa at a rate of 50 MPa/min. Afterwards, the pressure was kept constant for 15 min. Afterwards the pressure was released within a few seconds and the vessels were opened. The temperature of the pressure vessels was controlled by a
circulating thermostatic liquid (35 °C) from an external bath through the jackets surrounding the vessels. The temperature was not constant during the pressure treatment, due to adiabatic heating (maximum temperature if 43 °C) and cooling (minimum temperature of 15 °C) during pressure buildup and release, respectively. Samples were frozen after HPP.

**HPP in Combination with Enzyme Treatment**

When HPP was combined with enzyme treatment, the enzyme mixtures were added to the puree before the plastic pouches were sealed. Enzyme dosages were standardized on 100 nkat of PG activity for Biopectinase CCM (4.4 µL/g berries) and Pectinex Ultra SP L (3.4 µL/g berries) and on 100 nkat of endo-glucanase activity for Econase CE (6.0 µL/g berries), respectively. The commercial enzyme mixtures contain other enzymes as well (Buchert et al., 2005). After HPP, the samples were kept for 2 h at room temperature and atmospheric pressure to allow the enzymes to work further on their substrates and frozen at -23 °C afterwards. One control was kept in the vessel but was not pressurized (mash TP), one control was kept at room temperature and atmospheric pressure (mash RT), and one control was pressurized without the addition of enzyme (mash + HPP).

**Preparation of Alcohol Insoluble Solids (AIS) and Sequential Buffer Extraction**

Cell wall material was precipitated with 70 % aqueous ethanol at 50 °C (AIS) and sequentially extracted with 0.05 M sodium acetate buffer at pH 5.2 and 70 °C (hot buffer soluble solids, HBSS), with 0.05 M EDTA and 0.05 M sodium acetate in 0.05 M sodium oxalate at pH 5.2 and 70 °C (chelating agent soluble solids, ChSS), with 0.05 M sodium hydroxide at 0 °C (diluted alkali soluble solids, DASS), and with 6 M sodium hydroxide at 0 °C (concentrated alkali soluble solids, CASS). The remaining residue was included in the analysis as well (De Vries et al., 1981; Vierhuis et al., 2000).

**Sugar Composition.**

Sugar composition was determined after Saeman hydrolysis: after pre-hydrolysis using 72 % w/w sulfuric acid at 30 °C for 1h, the samples were hydrolyzed with 1 M sulfuric acid at 100 °C for 3 h (Saeman et al., 1945). Afterwards, the sugars were derivatised to alditol acetates and determined by gas chromatography (Englyst and Cummings, 1984) using inositol as internal standard.

**Uronic Acid Content.**

The total uronic acid content was determined photometrically with the automated m hydroxydiphenyl assay (Thibault, 1979).

**Degree of Methyl Esterification (DM) and Degree of Acetylation (DA).**

DM and DA were determined by HPLC after hydrolysis with 0.4 N sodium hydroxide in 2-propanol/water 50:50 v/v (Voragen et al., 1986). In addition, the DM was also determined by headspace gas chromatography (Huisman et al.,
Effect of combined enzymatic and high pressure processing on cell wall polysaccharides

DM and DA were calculated as moles of methyl/acetyl groups per 100 mol of galacturonic acid. One mole galacturonic acid can carry only 1 mol of methyl esters and 2 mol of acetyl ester.

Results

Effect of HPP on Cell Wall Polysaccharides.

To investigate if HPP changes the structure, composition, or extractability of cell wall polysaccharides, bilberries and black currants were mashed and treated at 400 MPa for 15 min, whereas the control remained at atmospheric pressure and room temperature. Cell walls present in these samples were isolated and their polysaccharide structure, composition, and extractability analyzed.

The sugar compositions of bilberry cell walls with and without HPP were very similar (table 6.1). No considerable differences in molar contents between the sugar residues were observed. The DM of pectins, however, was affected by HPP. It decreased after HPP of bilberry mash from 67 to 58 % in AIS. Such a decrease was similar in ChSS (from 86 to 73 %). In HBSS the DM remained remarkably high (96 and 106 %). Obviously, a DM above 100 % is theoretically impossible, but was shown with two different methods to measure methanol. We have no explanation for this aberrant value and consider the DM unchanged in HBSS.

The sugar composition of black currant AIS (table 6.2) did not change considerably after HPP. An exception is an unexplainable decrease of the mannose content in AIS and the residue. In HBSS of the HPP mash, mannose and glucose were present in higher molar contents compared to control HBSS. However, this was not due to an increase in these sugar contents, but to a decrease in uronic acid content (vide infra). Like in bilberries, the most remarkable change was the decrease of DM after HPP. This was shown for pectins present in AIS and ChSS, but not for pectins in HBSS.

With a decrease in DM, a change in extractability of uronic acid after HPP was observed. Figure 6.1 shows how much uronic acid is extracted from AIS into the different fractions. The highest amount of uronic acid was found in HBSS for all four mashes. In bilberry control mash, uronic acid was extracted into the ChSS and DASS fractions to the same extent, whereas the lowest content of uronic acid was extracted into CASS. After HPP, the content of uronic acid decreased in DASS, whereas it increased in ChSS. This means a better extractability of uronic acid after HPP, because ChSS is extracted under milder conditions than DASS. The high amount of uronic acid in the residue is due to pectic polysaccharides, which are known to be present in the seeds (Hilz et al., 2005) that remained intact during extraction.

An enhanced extractability of uronic acid was observed for HPP mash of black currants compared to black currant control mash, as well. The uronic acid content decreased in DASS and CASS from HPP mash of black currants, whereas the uronic acid content increased in HBSS and hardly changed in ChSS, which is in contrast to bilberry mash.
**Table 6.1** Composition of cell walls and cell wall fractions isolated from bilberry mash treated without (C = control) and with high pressure (HPP).

<table>
<thead>
<tr>
<th></th>
<th>yield per wet mash [g/100g]</th>
<th>[mol %]</th>
<th>total sugar residues [% w/w]</th>
<th>DM [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIS</td>
<td>5.1</td>
<td>0.2</td>
<td>0.4</td>
<td>47</td>
</tr>
<tr>
<td>HBSS</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>39</td>
</tr>
<tr>
<td>ChSS</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>29</td>
</tr>
<tr>
<td>DASS</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>33</td>
</tr>
<tr>
<td>CASS</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>47</td>
</tr>
<tr>
<td>residue</td>
<td>3.1</td>
<td>1.1</td>
<td>1.1</td>
<td>47</td>
</tr>
</tbody>
</table>

**Table 6.2** Composition of cell walls and cell wall fractions isolated from black currant mash treated without (C = control) and with high pressure (HPP).

<table>
<thead>
<tr>
<th></th>
<th>yield per wet mash [g/100g]</th>
<th>[mol %]</th>
<th>total sugar residues [% w/w]</th>
<th>DM [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIS</td>
<td>8.3</td>
<td>1.2</td>
<td>1.2</td>
<td>47</td>
</tr>
<tr>
<td>HBSS</td>
<td>0.8</td>
<td>1.1</td>
<td>1.1</td>
<td>39</td>
</tr>
<tr>
<td>ChSS</td>
<td>0.4</td>
<td>0.5</td>
<td>0.5</td>
<td>29</td>
</tr>
<tr>
<td>DASS</td>
<td>0.6</td>
<td>0.4</td>
<td>0.4</td>
<td>33</td>
</tr>
<tr>
<td>CASS</td>
<td>1.2</td>
<td>0.7</td>
<td>0.7</td>
<td>47</td>
</tr>
<tr>
<td>residue</td>
<td>4.6</td>
<td>1.1</td>
<td>1.1</td>
<td>47</td>
</tr>
</tbody>
</table>

**Table 6.3** Composition of cell walls (AIS) isolated from black currant mash treated with different commercial enzyme mixtures with and without HPP.

<table>
<thead>
<tr>
<th></th>
<th>[g/kg mash]</th>
<th>total sugar residues [% w/w]</th>
</tr>
</thead>
<tbody>
<tr>
<td>mash RT</td>
<td>0.8</td>
<td>46.5</td>
</tr>
<tr>
<td>mash TP</td>
<td>0.9</td>
<td>50.0</td>
</tr>
<tr>
<td>mash + HPP</td>
<td>0.8</td>
<td>53.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>[g/kg mash]</th>
<th>total sugar residues [% w/w]</th>
</tr>
</thead>
<tbody>
<tr>
<td>mash + Econase CE</td>
<td>0.4</td>
<td>34.8</td>
</tr>
<tr>
<td>mash + Econase CE + HPP</td>
<td>0.8</td>
<td>41.2</td>
</tr>
<tr>
<td>mash + Biopectinase CCM</td>
<td>0.3</td>
<td>28.3</td>
</tr>
<tr>
<td>mash + Biopectinase CCM + HPP</td>
<td>0.1</td>
<td>34.2</td>
</tr>
<tr>
<td>mash + Pectinex Ultra SP</td>
<td>0.3</td>
<td>31.7</td>
</tr>
<tr>
<td>mash + Pectinex Ultra SP + HPP</td>
<td>0.1</td>
<td>31.3</td>
</tr>
</tbody>
</table>
Combination of HPP and Treatment with Commercial Enzymes.

In order to test if HPP and commercial enzymes have a synergistic effect on degradation of cell wall polysaccharides, black currants were homogenized and three different commercial enzymes mixtures (Econase CE, Biopectinase CCM, and Pectinex Ultra SP L) were added directly before HPP.

Comparing the sugar composition of AIS indicates how enzymes affect cell wall polysaccharides (table 6.3). The sugar compositions of AIS did not differ significantly among the three controls. Treatment with Biopectinase CCM and Pectinex Ultra SP-L caused a decrease in the content of hemicellulolytic sugars (xylose, mannose, glucose) at atmospheric pressure compared to all three control samples, but not at high pressure.

Pectins behaved differently from hemicelluloses. The content of uronic acid in AIS decreased after treatment with Biopectinase CCM and Pectinex Ultra SP L. The uronic acid content after treatment at high pressure was even lower than after treatment at atmospheric pressure. The content of rhamnose decreased in AIS after treatment with Biopectinase CCM and Pectinex Ultra SP L at atmospheric pressure. After treatment at high pressure, the decrease was much stronger: almost 90 % of rhamnose was removed. The content of galactose and arabinose decreased in AIS after treatment with Biopectinase CCM and Pectinex Ultra SP L at atmospheric pressure and at high pressure. The decrease of galactose content was stronger after treatment at atmospheric pressure (>50 %), whereas the arabinose content decreased equally after treatment at atmospheric and high pressure.

![Figure 6.1 Content of uronic acids in the different cell wall fractions obtained from mashes treated and non-treated with HPP [% of extracted uronic acid.]](image-url)
The sample treated with Econase CE showed a decrease of hemicellulolytic sugars (xylose, mannose, glucose) after treatment at atmospheric pressure. If Econase was used in combination with high pressure, there was hardly any change in the content of hemicellulolytic sugars. The different pectic sugar residues showed similar behavior as after treatment with the pectinolytic enzyme mixtures, but to a lesser extent.

A more detailed picture of the nondegradable polysaccharides gives their extractability to HBSS and ChSS (table 6.4). In the control (mash TP) only a minor part of the hemicellulolytic sugars (xylose, mannose, glucose) was extracted to HBSS and ChSS (2–5%) and ~15% of the neutral pectic sugar residues (rhamnose, galactose, arabinose) were extracted with these two agents.

Table 6.4 shows data for the degradation with Biopectinase CCM. The data for Pectinex Ultra SP L were similar (not shown). Data for Econase CE are not shown, because this enzyme mixture is mainly active on hemicelluloses, which are not extracted with hot buffer or chelating agent. Although only a minor part of the hemicellulososes was extracted in the pectic fractions (HBSS and ChSS), a decreased extractability of hemicelluloses was observed after treatment with Biopectinase CCM at high pressure.

The uronic acid that remained after treatment with Biopectinase CCM at atmospheric pressure was more easily extractable from AIS with hot buffer and chelating agent compared to the control mash, but after treatment at high pressure, uronic acid was mainly extracted with chelating agent. Apparently, enzymes solubilized tightly bound uronic acids and degraded easily extractable pectins (HBSS). The pectin remaining after HPP was mainly calcium bound (ChSS). Rhamnose was removed after treatment with Biopectinase CCM, and the remaining rhamnose was extracted into HBSS. Compared to the control, relatively more of the remaining galactose residues were extracted to HBSS after treatment at atmospheric pressure, whereas no differences in relative extractability were observed after HPP. From the remaining arabinose a higher percentage was extracted with hot buffer after treatment at atmospheric pressure. More tightly bound pectins, probably rhamnogalacturonan I with arabinan and arabinogalactan side chains, were degraded at atmospheric pressure.

The DM decreased in AIS and ChSS and remained the same in HBSS after HPP of black currant control mash (table 6.5). This is similar to the results from the previous trial (table 6.2). No change in DM was observed when the control mash followed the same temperature profile as the HPP control. The DA differed in the three controls (table 6.5). It decreased in AIS at higher temperature of the treatment, but not after HPP. The DA did not change with temperature or pressure in the pectic fractions (HBSS, ChSS). Thus, the change in acetylation probably occurred in hemicellulososes (vide infra).

The DM decreased in AIS after treatment with Biopectinase CCM at atmospheric pressure and at high pressure even more. The DM was also drastically reduced in HBSS and ChSS after treatment with Biopectinase CCM at high pressure. The DA increased in AIS and HBSS after treatment with
Effect of combined enzymatic and high pressure processing on cell wall polysaccharides

Biopectinase CCM. This increase was higher after treatment at high pressure. Because the DA is calculated per mole of uronic acid, this increase may correlate with the decrease of the uronic acid content. Whether the remaining uronic acid was acetylated is not clear. Acetyl groups could also be attached to hemicelluloses.

Table 6.4 Sugar composition of black currant AIS and extractability to HBSS and ChSS of cell wall polysaccharides in control sample and samples treated with Biopectinase CCM at atmospheric and high pressure.

<table>
<thead>
<tr>
<th></th>
<th>Rha</th>
<th>Fuc</th>
<th>Ara</th>
<th>Gal</th>
<th>uronic acid</th>
<th>Xyl</th>
<th>Man</th>
<th>Glc</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong>: mash TP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIS [g/kg mash]</td>
<td>0.9</td>
<td>0.1</td>
<td>3.0</td>
<td>3.0</td>
<td>15.3</td>
<td>2.6</td>
<td>12.8</td>
<td>12.3</td>
<td>49.9</td>
</tr>
<tr>
<td>HBSS [% sugar residue relative to AIS]</td>
<td>11</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>18</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>ChSS [% sugar residue relative to AIS]</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td><strong>B</strong>: Biopectinase CCM</td>
<td></td>
<td></td>
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<tr>
<td>AIS [g/kg mash]</td>
<td>0.3</td>
<td>0.1</td>
<td>1.2</td>
<td>1.5</td>
<td>6.4</td>
<td>1.9</td>
<td>7.3</td>
<td>9.6</td>
<td>28.4</td>
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<tr>
<td>HBSS [% sugar residue relative to AIS]</td>
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<td>0</td>
<td>31</td>
<td>24</td>
<td>37</td>
<td>5</td>
<td>7</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>ChSS [% sugar residue relative to AIS]</td>
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<td>0</td>
<td>7</td>
<td>3</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td><strong>C</strong>: Biopectinase CCM + HPP</td>
<td></td>
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<tr>
<td>AIS [g/kg mash]</td>
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<td>0.1</td>
<td>1.5</td>
<td>2.2</td>
<td>3.7</td>
<td>2.2</td>
<td>12.6</td>
<td>11.8</td>
<td>34.4</td>
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<tr>
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<td>4</td>
<td>7</td>
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<td>3</td>
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<td>3</td>
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<tr>
<td>ChSS [% sugar residue relative to AIS]</td>
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<td>0</td>
<td>2</td>
<td>2</td>
<td>28</td>
<td>1</td>
<td>1</td>
<td>1</td>
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Table 6.5 Degree of methyl esterification (%DM) and acetylation (%DA) in black currant mash treated with different commercial enzyme mixtures with and without HPP.

<table>
<thead>
<tr>
<th></th>
<th>AIS</th>
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<th>ChSS</th>
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<td>DA</td>
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<tr>
<td>mash + Econase</td>
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<td>102</td>
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<td>mash + Econase + HPP</td>
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<td>73</td>
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<td>36</td>
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<tr>
<td>mash + Biopectinase CCM + HPP</td>
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<td>6</td>
</tr>
<tr>
<td>mash + Pectinex Ultra SP-L</td>
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<td>49</td>
<td>60</td>
</tr>
<tr>
<td>mash + Pectinex Ultra SP-L + HPP</td>
<td>23</td>
<td>51</td>
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</table>
Discussion

Effect of HPP on Pectin Structure in Berries

Our current study gives an overview of the polysaccharide composition of cell walls and cell wall fractions in bilberries and black currants before and after HPP. From this overview it can be deduced if HPP has an impact on berry processing. Cell walls are important for the texture of berries. High pressure was not applied on whole berries but on berry mash to have a more homogeneous sample.

The main components that influence the structure and viscosity of berry mash are pectic polysaccharides. After mashing, a highly viscous pectin gel is formed, which hinders pressing and reduces the juice yield (Grassin and Fauquembergue, 1996). After HPP, pectic polysaccharides lose methyl esters and, therefore, get more free carboxyl groups. The DM decreases. This results in a higher calcium sensitivity of the pectins (Ralet et al., 2003) and, therefore, formation of a stronger pectin gel after HPP, which hampers the release of juice. HPP of tomato products results also in a firmer texture and higher viscosities (Porretta et al., 1995; Fernandez Garcia et al., 2001; Krebbers et al., 2003). This is mainly related to the increased activity of pectin methyl esterase (PME), which is supposed to result in a decreased DM (Hernandez and Cano, 1998; Krebbers et al., 2003). Unfortunately, in these studies the DM of tomato pectins was not determined. A decreased DM accompanied by a firmer texture of vegetables was reported for carrots (Kato et al., 1997) and Japanese radish (Yamamoto et al., 1992) after HPP. PME activity of Japanese radish, however, remains the same before and after HPP (Yamamoto et al., 1992). HPP of synthetically amidated, low methyl esterified pectin solutions gives a stronger gel (Abbasi and Dickinson, 2002). Amide groups are known to enhance the hydrogen bonds (Alonso-Mougan et al., 2002), which are particularly affected by HPP (Farr, 1990).

It has been suggested that pectic polysaccharides become easier to extract after HPP (Krebbers et al., 2003). Our results show this clearly. In the first instance it was expected that a decrease in DM would occur in easily extractable and, therefore, easily accessible pectins. This would cause a shift of pectins from HBSS to ChSS (calcium sensitive fraction). However, our results show a different effect. In bilberries the amount of pectic polysaccharides in HBSS remained the same, whereas it increased in HBSS of black currants. A decrease was observed only in DASS. The DM remained the same in HBSS and decreased only in ChSS. This is in contrast to the findings for guava juice, for which the DM remains unchanged in all pectic populations after HPP (Yen and Lin, 1998). Surprisingly, these authors found the highest DM in DASS, which is very unlikely and raises doubts about the analytical methodology, because alkali saponifies the methyl esters. In mashies of bilberries and black currants, HPP seems to cause modifications in specific populations within the pectic polysaccharides. This is probably an effect of high pressure on pectinolytic enzymes. A possible
Effect of combined enzymatic and high pressure processing on cell wall polysaccharides

An explanation would be that HPP favors the formation of complexes between enzymes such as PME and pectin substrate, which is deposited in the cell wall as calcium complexes or as complexes with the hemicellulose-cellulose network through hydrogen bonds.

**Effect of HPP on Commercial Enzymes**

Processing berry mashes with high pressure improves extractability and increases calcium sensibility, leading to a stronger pectin gel. During juice production commercial enzyme mixtures containing mainly pectinolytic enzymes of fungal origin are used to degrade such a pectin gel (Grassin and Fauquembergue, 1996). Combining HPP and treatment with commercial enzyme mixtures might have a synergistic effect on the degradation of cell wall polysaccharides. This would lead to a higher juice yield and a better extractability of phenolic compounds into the juice (Landbo and Meyer, 2001; Meyer, 2002; Bagger-Jorgensen and Meyer, 2004; Buchert et al., 2005). At the same time minor enzyme activities present in commercial enzyme mixtures might be activated and affect cell wall polysaccharides differently than at atmospheric pressure.

Our results show that hemicellulases were active only if the enzymatic treatment is carried out at atmospheric pressure. If high pressure was applied to the sample immediately after the addition of the enzymes, no degradation of hemicelluloses was observed. Apparently, hemicellulases are inactivated by the high pressure applied. This has been shown for polygalacturonases (PG) from different plants as well (Ludikhuyze et al., 2003; Fachin et al., 2004; Peeters et al., 2004; Verlent et al., 2004).

High pressure did not seem to inactivate exogenous pectinolytic enzymes in the berry mash matrix. This is in contrast to results of isolated plant PG, which are very quickly inactivated with increasing pressure (Fachin et al., 2004; Peeters et al., 2004; Verlent et al., 2004). Commercial pectinolytic enzyme mixtures are mostly of fungal origin. It was expected that they would be inactivated by HPP just like plant PGs. Perhaps fungal PGs differ structurally from plant PGs and are pressure resistant up to 400 MPa. Although similarities in tertiary structure can be observed between fungal and plant PGs, there are major differences in the loop regions (Benen and Visser, 2003). Another explanation might be that soluble solids in the berry mash protect PG against inactivation, as seen for isolated PME under pressure (Ogawa et al., 1990). The carrot matrix is able to protect PME under pressure as well (Balogh et al., 2004).

However, added pectinolytic enzymes appeared not only pressure stable; in most cases even a further degradation of pectins was observed after HPP. Degradation of uronic acid residues present in homogalacturonan is dependent not only on PG activity but on PME or pectin lyase (PL) activity as well. PG can degrade homogalacturonan only if it is deesterified for example by PME. PL degrades only methyl-esterified homogalacturonan. Homogalacturonan degradation was observed after treatment with Biopectinase CCM and Pectinex Ultra SP L at atmospheric pressure. HPP decreased the content of uronic acid.
even further. This activation can have several causes. First of all, we showed a 
decrease of the DM in AIS after HPP of berry mash. A lower DM facilitates the 
action of PG. Next to endogenous PME, PME was added to the mash by adding 
commercial enzyme mixtures. Although this PME is from fungal origin, it might 
also be activated under high pressure in a similar way as plant PME (Ogawa et 
al., 1990; Seyderhelm et al., 1996; Ludikhuyze et al., 2003; Balogh et al., 2004). 
The observed further decrease of the DM after HPP supports this possibility. 
From our results it appears that PG retains its activity up to 400 MPa. It might 
even be activated under high pressure and would then be able to degrade the 
substrate further during the incubation time after HPP. Another option is the 
formation of an energetically beneficial complex between enzyme and substrate 
under high pressure, resulting in more degradation when both substrate and 
enzyme are present during HPP. In this case PG activity may not necessarily 
change after the pressure is released.

The content of rhamnose, which is mainly present in the pectic polysaccharide 
rhamnogalacturonan I, decreased after treatment with Biopectinase CCM or 
Pectinex Ultra SP L at atmospheric pressure and decreased even further after 
treatment at high pressure. This is an indication for an activation of 
rhamnogalacturonan I degrading enzymes such as rhamnogalacturonases 
present in these two enzyme mixtures (Schols et al., 1990a). 
Rhamnogalacturonan I was degraded into small alcohol soluble oligomers that 
are removed during alcohol extraction. The question of whether improved 
degradation of rhamnogalacturonan I is really due to activation of the enzyme or 
improved accessibility cannot be answered, although such a difference between 
treatments at atmospheric pressure and at high pressure is a strong indication 
for enzyme activation.

The DA increased after treatment with pectinolytic enzymes and even more 
when in combination with HPP. This increase in DA correlates with the decrease 
of uronic acid. Because the DA was calculated per mole of uronic acids, a decrease 
in uronic acid content while retaining the acetyl groups results in an increased 
DA. The amount of acetyl esters present did not change. We assumed before that 
a significant part of the acetyl substitution is on the hemicelluloses (Hilz et al., 
2005), Why the DA decreases in the control when the same temperature profile is 
followed as under HPP is, however, not clear.

If high pressure is applied to bilberry or black currant mash, to which a 
commercial enzyme mixture is added, hemicelluloses are not affected, probably 
due to an inactivation of hemicellulases. Pectic polysaccharides (mainly 
homogalacturonan and rhamnogalacturonan I) are, however, degraded to a larger 
extent compared to treatment at atmospheric pressure. Thus, treatment with 
commercial enzymes in combination with controlled HPP opens possibilities to 
tailor enzymatic modification of polysaccharides and, therefore, the texture or 
other properties such as extractability of phenolic compounds into juice. Detailed 
analysis of juice yields and polyphenol composition of juices produced by HPP 
will be reported later. Our study included only one condition for the HPP 
treatment that was shown to be effective in preliminary experiments.
Nevertheless, evidence is presented that HPP has the potential to affect cell wall polysaccharides, particularly in combination pectinolytic enzymes, possibly improving processing of bilberries and black currants.

Acknowledgment

We thank Xiaowei Zheng, Wageningen University, for her contribution within her bachelor’s degree thesis and Vernu Vasunla, Kiantama Ltd., Finland, for providing us with commercial berry material.

This study has been carried out with financial support from the Commission of the European Communities, specific RTD program ‘Quality of Life and management of Living Resources’, contract number QLK1-CT-2002-02364 ‘Novel enzyme-aided extraction technologies for maximized yield and functionality of bioactive components in consumer products and ingredients from by-products’, acronym MAXFUN. It does not necessarily reflect its views and in no way anticipates the Commission’s future policy in this area.
Chapter 7

Concluding Remarks
Motivation of the research

Cell wall polysaccharides influence processability of black currants and bilberries during juice production. In a first processing step berries are mashed. During this mashing cell walls are disrupted and cell wall polysaccharides, especially pectins, are released and form a strong gel (Grassin and Fauquembergue, 1996). To degrade this pectin gel pectolytic enzyme mixtures of fungal origin are added to the mash (Grassin and Fauquembergue, 1996). Pectins are degraded, viscosity decreases, and, therefore, juice yields increase. Furthermore, by the application of commercial enzymes preparations the content of phenolic substances in the juice increases (Landbo and Meyer, 2001; Meyer, 2002; Bagger-Jorgensen and Meyer, 2004). This can partly be explained by degradation of cell walls of epidermis cells, which contain anthocyanins within their vacuoles. After destruction of the cell walls the content of the vacuoles is then able to leach into the mash (Ortega-Regules et al., 2006). It is also possible that polyphenols are entrapped in the cell wall matrix and are released after their degradation (Bagger-Jorgensen and Meyer, 2004). Anthocyanins are together with other flavonoids the colorants of the berries and are discussed to have health promoting properties due to their antioxidative capacity (Baj et al., 1983; Morazzoni and Bombardelli, 1996; Heinonen et al., 1998a; Heinonen et al., 1998b; Hou, 2003).

Although enzyme preparations have been used by the berry processing industry since the 1930s (Grassin and Fauquembergue, 1996), the commercial enzyme preparations were designed mainly using empirical data of juice yield and juice quality. These parameters are also influence by the kind of press used in combination with the enzyme preparations. Hardly any work has been done to identify cell wall components of black currants or bilberries and their structures. For an optimal application and the choice of the best commercial enzyme preparations, structural information about the cell wall components, viz. the polysaccharides cellulose, hemicelluloses, and pectins, is needed.

Carried out within the EU-project ‘Novel enzyme-aided extraction technologies for maximised yield and functionality of bioactive components, products, and ingredients from by-products’, acronym MAXFUN, the aim of this thesis was to characterise the structural elements of the plant cell wall. The MAXFUN-project aimed at improvements in juice processing by using novel enzymatic and physical treatments. A better understanding of the cell wall composition enables also monitoring of cell wall components during processing. Not only the effect of conventional juice production, but also the use of high pressure with or without addition of commercial enzymes preparations was investigated in this study with respect to changes in cell wall composition and enzymatic degradability.
Cell wall polysaccharides of black currants

Within this thesis pectins and hemicelluloses of black currants were analysed. The structures of pectins and hemicelluloses were determined and the extractabilities of the different cell wall polysaccharides were shown. Furthermore, changes in pectins and hemicelluloses during processing were monitored.

Pectic polysaccharides of black currants

Characteristics of pectins present in black currant are described in chapter 2. In cell wall material of black currants, pectic galacturonic acids were the main sugar residues and contribute with almost 45% to the total sugar residues. More arabinose residues were present than galactose residues. Two times more homogalacturonan (‘smooth regions’) was present than rhamnogalacturonan I including the neutral sugar side chains (‘hairy regions’). Both skin and pulp were rich in pectins and more than half of the total sugar residues were pectic residues in these tissues. Due to the higher content of pulp compared to skin, most of the pectins of black currants were located in the pulp. Hemicelluloses were prevailing in the seeds, but some pectic polysaccharides were also present.

By fractionation of the cell wall polysaccharides, different types of pectins were separated from hemicelluloses and celluloses. Contents and properties of the polysaccharides in the different fractions allowed a detailed insight into the cell wall composition of black currants. Most of the pectins were extracted from the cell wall material with hot buffer. Calcium sensitive pectins were solubilised with hot buffer containing chelating agent. The remaining pectin was released from the (hemi)celluloses with diluted alkali solution. These three fractions contained almost exclusively pectins. While pectins extracted with hot buffer with or without chelating agent consisted for more than 80% of homogalacturonan, pectins extracted with diluted alkali contained higher levels of rhamnogalacturonan I, but still mainly homogalacturonan. With 6 N sodium hydroxide hemicelluloses were extracted, while hardly any pectic galacturonic acid residues were present in this fraction. Some pectic polysaccharides remained in the residues. These pectins were located in the seeds, which remained intact during extraction. The ratio of homogalacturonan to ramified rhamnogalacturonan I in the seeds was probably 1:1. This is only an estimated value, because galactose and arabinose residues, which are the sugar residues present in the neutral side chain, were present in hemicelluloses, as well. To make the interpretation of the results with respect to hemicelluloses and pectic sugars easier and to prevent extraction of polysaccharides from the exosperm into the fractions, in future experiments it would be advantageous to separate seeds from the rest of the berry material. By this only polysaccharides of the soft tissues, which have the highest influence on the physical behaviour of berries and berry products, will be analysed. The molecular weight of the polysaccharides present in the various fractions decreased with the harshness of the extraction conditions. Hot buffer
extracted pectins had a higher molecular weight than the pectins extracted with diluted alkali. The sequential extraction starts with non-degrading hot buffer. Whether hydrolysis of ester bonds under alkali condition is responsible for the decrease in molecular weight, is not clear. Hydrolysis of uronyl esters would explain a decrease in molecular weight, but their existence has never been proven (see chapter 1 and Mort, 2002). More probable is, however, that native pectins with lower molecular weight are extracted with diluted alkali. A degradation by β-elimination was prevented by decreasing the extraction temperature to 0 °C and using reducing condition by addition of sodium borohydrate.

Chapter 3 describes the presence of rhamnogalacturonan II (RG II) in black currants. All pectic fractions contained between 7% and 8% RG II. This RG II was incorporated into the pectin structure and was released by polygalacturonase in combination with pectin methyl esterase or pectin lyase. The released RG II was shown to be esterified to the dimeric form. RG II probably connects two different pectin chains, intra- or intermolecular and is, thus, an important cross link in the cell walls of black currants.

In chapter 2 the esterification of the polysaccharides in black currants is described, as well. The degree of methyl esterification of the pectins present in cell wall material was 55%. The pectins with the highest degree of methyl esterification were extracted with hot buffer. Although the pectins with a degree of methyl esterification lower than 50% are considered as calcium sensitive (Ralet et al., 2003), pectins extracted with chelating agent, which complexes the calcium ions, had still a degree of methyl esterification of 58%. The methyl esters were probably distributed blockwise over the pectic homogalacturonan chains. Calcium sensitivity has been shown before for a commercially available high methyl esterified pectin with a blockwise distribution of the methyl groups (Guillotin et al., 2005). The degree of acetylation was low in black currants. Although the degree of acetylation was calculated as galacturonic acid residues esterified with acetyl groups, it is possible that hemicelluloses are esterified with acetyl groups, as well. If all acetyl groups were connected to pectins, was not investigated.

**Hemicelluloses of black currants**

The sugar linkage composition of hemicelluloses in chapter 2 shows that the main hemicelluloses of black currants were mannans. From the mannose present in the cell wall material of the whole berries, 95% was found in the seed, where they are the dominating sugar residues. The mannose residues were 1,4-linked, but a detailed characterisation was not performed within this thesis. The mannans probably function as storage polysaccharides.

In the cell wall material of black currants, xyloglucans were identified as described in chapter 4. After an isolation procedure by anion exchange chromatography, the sugar composition of the partly purified xyloglucan fraction was determined. Sugar residues present were glucose and xylose and, to a lesser extent, galactose and fucose. The xyloglucans of black currants were degraded
Chapter 7

with xyloglucan specific endo-glucanase in the cell wall material, in the hemicellulose fraction, and in different fractions obtained by anion exchange chromatography. Obtained oligomers were analysed using various techniques. To simplify the nomenclature of xyloglucan oligomers, the different side chains were assigned one-letter codes (Fry et al., 1993). Some examples for the letter codes are G for an unsubstituted β-D-glcp, X for an α-D-xylp-(1→6)-β-D-glcp unit, L for a β-D-galp-(1→2)-α-D-xylp-(1→6)-β-D-glcp unit, or F for an α-L-fucp-(1→2)-β-D-galp-(1→2)-α-D-xylp-(1→6)-β-D-glcp unit. Two main structures of xyloglucans are known: XXXG-type and XXGG-type (Vincken et al., 1997). Black currant xyloglucans had XXXG-type structure. They contained galactose and fucose in the side chains (L and F, respectively). Three main building blocks were XXXG, XXFG, and XLFG. Other building blocks present were XXG, XLXG, XXLG, and XLLG. The galactose residues of the L- or F-unit were partly acetylated.

The structure of black currants xyloglucans is similar to xyloglucan structures of many other fruits and vegetables (Hoffman et al., 2005).

**Cell wall polysaccharides of bilberries**

Next to black currant polysaccharides, also bilberry pectins and hemicelluloses were analysed during the study described within this thesis. The structure and extractability of the polysaccharides was determined in a similar approach as for black currants. Based on the obtained data, changes of the cell wall polysaccharides during processing of the berries were monitored.

**Pectic polysaccharides of bilberries**

In chapter 2 the general composition of bilberry pectins is described. Pectic galacturonic acid residues were present in lower amounts in bilberries compared to black currants. The main residues of cell wall polysaccharides in bilberries were glucose and xylose. Galacturonic acid residues represented 24 % of the total sugar residues, which is half of relative amount of galacturonic acid residues in black currants. Arabinose and galactose residues were present in small amounts to the same molar ratios. Two times more homogalacturonan (‘smooth regions’) was present than rhamnogalacturonan I including neutral arabinan and arabinogalactan side chains (‘hairy regions’). Comparable to black currants, skin and pulp were rich in pectins. Almost half of the total sugar residues were pectic residues in these tissues (49 %), which was lower than in black currants (65 %). Due to the higher content of pulp compared to skin, most of the bilberry pectins were located in the pulp. However, most of the cell wall polysaccharides of bilberries originated from the seeds. Although the hemicelluloses xylans and xyloglucans were the prevailing polysaccharides in bilberry seeds, some pectins were also present.

Cell wall polysaccharides of bilberries were fractionated in the same way as those of black currants. With hot buffer followed by hot buffer containing chelating agent and 50 mM sodium hydroxide, almost exclusively pectins were
Concluding Remarks

extracted. While pectins extracted with hot buffer with or without chelating agent consisted for more than 80 % of homogalacturonan, pectins extracted with diluted alkali contained more rhamnogalacturonan I, but homogalacturonans formed the majority. Most of the pectins were extracted with hot buffer. Hemicelluloses were extracted with 6 N sodium hydroxide. Hardly any pectic galacturonic acid residues were present in this fraction. As in black currants, some pectic polysaccharides remained in the residues. These pectins were located in the seeds, which remained intact during extraction. The ratio of homogalacturonan to ramified rhamnogalacturonan I of pectins present in the seeds was estimated to be 1:1. Similar to the results obtained for black currants, the molecular weight of the polysaccharides present in the different fractions decreased with the harshness of the extraction solution. This is probably not because of degradation during the extraction procedure, but because of extractability of the native pectins with lower molecular weight.

In chapter 3 determination of RG II in bilberries is described. In all pectic fractions of bilberries RG II represents between 5 and 10 % of the total polysaccharides. This RG II was present as a dimer and incorporated into the pectin structure. It was released from the pectic polymer by polygalacturonase digestion in combination with pectin methyl esterase.

Quantification of methyl and acetyl esters present in cell wall polysaccharides of bilberries is included in chapter 2. The degree of methyl esterification of the pectins present in cell wall materials was 60 %. The pectins with the highest degree of methyl esterification were extracted with hot buffer. Pectins extracted with chelating agent, had still a degree of methyl esterification of 70 %, although the pectins with a degree of lower than 50 % are considered as calcium sensitive (Ralet et al., 2003). But a blockwise distribution can cause a high methylated pectin to become calcium sensitive (Guillotin et al., 2005). Thus, the methyl esters were probably distributed blockwise over the homogalacturonan chains. In the same fraction of black currants 58 % of the galacturonic residues of homogalacturonan were esterified with methanol. The degree of acetylation was very high in bilberries. After determining the degree of acetylation in the different fractions, it was shown that mainly hemicelluloses were esterified with the acetyl groups, although the degree of acetylation was calculated as acetyl groups per galacturonic acid residues.

Bilberries contain less pectins than black currants and these pectins have a higher degree of methyl esterification. While pectins are the major polysaccharides in black currants, bilberries are dominated by hemicelluloses located in the seeds.

Hemicelluloses of bilberries

The sugar composition analysis of the different fractions and tissues in chapter 2 shows that xylans and xyloglucans were the main hemicelluloses of bilberries. Hardly any mannans, which are the dominating hemicelluloses in black currant seeds, were present. The xylans were mainly located in the seeds, where they function as storage polysaccharides. A high amount of xylose residues
were found in the pulp, as well. These xylose units were attributed to xyloglucans of the primary cell wall. We refrained from a detailed structural characterisation of storage xylans, but focused on xyloglucans of bilberries instead.

In chapter 5 the structure of xyloglucans from bilberries is described. In cell wall material and in the hemicellulose fraction obtained from it by alkali extraction after removal of the pectins the xyloglucans were degraded by xyloglucan specific endo-glucanase and the obtained building blocks were identified. More than 20 building blocks were released from bilberry xyloglucans by treatment with xyloglucan specific endo-glucanase. The basic structure of bilberry xyloglucans was XXXG-type xyloglucan, but some XXG-type building blocks were identified, as well. Fucose and galactose containing side chains (L and F) were shown in the some building blocks. In these L- and F-side chains, the galactose residue was partly acetylated. Interestingly, some xyloglucan oligomers contained a side chain of two pentoses. By chromatographic, electrophoretic, and mass spectrometric determination, the presence of a β-xylose attached to an α-xylose unit (U-side chain) was suggested and was proven after isolation and 1H NMR analysis of four building blocks. This side chain was described before in the argan tree (Ray et al., 2004), another member of the Ericales order, which also includes bilberries. Next to the three building blocks XUXG, XULG, and XUFG already described in the xyloglucans form the argan tree, four novel building blocks were identified in bilberries: XUG, XUUG, XLUG, and XXUG. Not only the high number of oligomers released from xyloglucans of bilberries, but also the presence of the U-chain in addition to L- and F-side chains make bilberry xyloglucans the xyloglucans with most complex structure described in the plant kingdom until today. Furthermore, bilberries are the second member of the Ericales order analysed for xyloglucan structure. Both species contained the U-side chain in their xyloglucan structure, what leads to the assumption that the U-side chain is a general structural element of xyloglucans in the Ericales family.

**Cell wall polysaccharides in other berries including grapes**

Detailed characterisation of cell wall polysaccharide of other berries is hardly described in literature. The best described berry cell wall is the one of grape berries (*Vitis vinifera* L.).

**Pectic polysaccharides of other berries**

The content of pectins in different berries was determined in literature. The level of pectins is high in black currants compared to other berries (table 7.1). Only cloudberries have higher pectin contents (Salo and Suomi, 1972). Red currants, gooseberries, black berries, and raspberries have pectin levels comparable to bilberries (Salo and Suomi, 1972; Krause and Bock, 1973). The pectins are located in the seedless part, viz. the pulp and the skin, of the berries. In these tissues more uronic acid than neutral sugars are found in the cell wall material (Salo and Suomi, 1972).
Table 7.1 Occurrence and proportions of the various structural elements of berries compared to other fruits.

<table>
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<th>black currant</th>
<th>bilberry</th>
<th>grape&lt;sup&gt;a&lt;/sup&gt;</th>
<th>strawberry&lt;sup&gt;b&lt;/sup&gt;</th>
<th>blueberry&lt;sup&gt;b&lt;/sup&gt;</th>
<th>raspberry&lt;sup&gt;b&lt;/sup&gt;</th>
<th>cloudberry&lt;sup&gt;b&lt;/sup&gt;</th>
<th>lingonberry&lt;sup&gt;b&lt;/sup&gt;</th>
<th>cranberry&lt;sup&gt;b&lt;/sup&gt;</th>
<th>soybean&lt;sup&gt;c&lt;/sup&gt;</th>
<th>sugar-beet&lt;sup&gt;c&lt;/sup&gt;</th>
<th>apple&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>total polysaccharides [% of dry matter]</strong></td>
<td>19</td>
<td>12</td>
<td>11</td>
<td>16</td>
<td>17</td>
<td>19</td>
<td>33</td>
<td>12</td>
<td>14</td>
<td>16</td>
<td>67</td>
<td>20</td>
</tr>
<tr>
<td><strong>pectic substances [% of total PS]</strong></td>
<td>61</td>
<td>33</td>
<td>56</td>
<td>56</td>
<td>46</td>
<td>39</td>
<td>35</td>
<td>61</td>
<td>58</td>
<td>59</td>
<td>40</td>
<td>42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>structural element [% of pectic substances]</strong></th>
<th>homogalacturonan</th>
<th>xylogalacturonan</th>
<th>rhamnogalacturonan I</th>
<th>rhamnogalacturonan II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>neutral side chains</strong></td>
<td>24</td>
<td>27</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>31</td>
<td>26</td>
<td>34</td>
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</tr>
<tr>
<td></td>
<td>24</td>
<td>31</td>
<td>26</td>
<td>35</td>
</tr>
</tbody>
</table>

n.a. = not analysed

<sup>a</sup> recalculated from Nunan et al. (1997)

<sup>b</sup> recalculated from Salo and Suomi (1972)

<sup>c</sup> from Voragen et al. (2001)

<sup>d</sup> recalculated from Vidal et al. (2001)
Grape berries have been characterised according to their tissue distribution (Lecas and Brillouet, 1994; Nunan et al., 1997; Vidal et al., 2001). Pulp and skin of grape berries contain uronic acids as the major sugar residues of their cell wall polysaccharides. The content is similar to bilberries, but lower compared to black currants. The ratio of homogalacturonan to rhamnogalacturonan I is with 2.5 to 1 higher than in the berries described in this thesis (table 7.1). Grape pulp contains relatively more rhamnogalacturonan II and less rhamnogalacturonan I and homogalacturonan than the skin. The proportions of the various structural elements in the berries are compared to other fruits, whose proportions were already described (Voragen et al., 2001). Berries including grape berries contain high amounts of homogalacturonan in comparison to other fruits. The ratio of homogalacturonan (‘smooth regions’) to rhamnogalacturonan I including the neutral side chain (‘hairy regions’) is approximately 2:1 in berries.

For berries the presence of pectins in skin and pulp seems very important. It is a common feature of all berries analysed until now. Pectic polysaccharides have a high water-binding capacity and can be easily modified by enzymes during ripening. Therefore, it is possible for a plant to produce relatively soft berries with a high water content.

**Hemicelluloses of other berries**

Seeds of different berries are rich in neutral sugars and have a lesser content in uronic acids (Salo and Suomi, 1972). Red currants and gooseberries contain mannose as major storage polysaccharides in the seeds similar to black currants. Most of the other berries such as raspberries, cloudberry, and strawberries contain a high content of xylose in their cell wall material (Salo and Suomi, 1972) comparable to bilberries as described in this thesis. Surprisingly, lingonberries and cranberries are rich in arabinose (Salo and Suomi, 1972). Maybe arabinose rich arabinogalactans function as storage polysaccharides in these berries.

Berries contain polysaccharides as energy source in their seeds. These polysaccharides are mainly hemicelluloses or arabinogalactan proteins. The type of polysaccharide present depends on the berry.

**The plant cell wall architecture**

Although the structural elements and polysaccharide components of the cell wall are pretty well known today, the architecture of the cell wall is still a riddle. Even the organisation of pectins has been discussed for years. Two models of pectins exist today. The older model describes pectins as a molecule composed of linear homogalacturonan regions, so called ‘smooth regions’, interrupted by ramified regions of a rhamnogalacturonan I backbone with arabinan and arabinogalactan side chains, so called ‘hairy regions’ (De Vries et al., 1982; De Vries, 1988). Basis of this model is a linear backbone of pectin, which contains only galacturonic acid residues interrupted by regions, where galacturonic acid and rhamnose alter. Since the early 21st century a second model has been discussed describing homogalacturonans as side chains of rhamnogalacturonan I
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(Vincken et al., 2003). In this model pectins are described as having a backbone of altering rhamnose and galacturonic acid residues to which neutral and acidic side chains are attached. Until today no unambiguous proof has been presented for any of the two models (Coenen, 2006).

The role of RG II in the cell wall is not completely understood. RG II can be released from the pectic macromolecules by treatment with polygalacturonase (Ishii and Matsunaga, 2001). This indicates that RG II is covalently linked to homogalacturonan. If this is the case, RG II is most probably present in the middle of the homogalacturonan chain, because the backbone of RG II is a galacturonic acid chain similar to homogalacturonan. It is also possible that RG II is present at the reducing end of a homogalacturonan chain between the homogalacturonan and rhamnogalacturonan I. The non-reducing end of homogalacturonan must be unsubstituted, because exo-polygalacturonase is able to degrade homogalacturonan from this non-reducing end (Saito, 1955; Guillon et al., 2006). However, it should be mentioned that it is also possible that RG II is incorporated into a homogalacturonan network and is released after degradation of homogalacturonan without being covalently linked to it. The further evidence for a covalent linkage presented by Ishii and Matsunaga (2001) is circumstantial. The authors obtained lower molecular weight pectin when the pectin was treated under conditions that are known to hydrolyse the boron ester. When this fraction was treated with boric acid in the presence of strontium to possibly reesterify RG II monomers, the original molecular weight was obtained. These observations might be caused by strontium-pectin interactions. Strontium is known to form strong complexes with pectins (Kohn and Tibensky, 1971).

Not only the linkages between the pectic elements are unknown, also the arrangement of the main polysaccharides pectins, hemicelluloses, and cellulose within the cell wall are still under discussion. Different models of the plant cell wall coexist and are adapted to newly presented evidence (Keegstra et al., 1973; McCann and Roberts, 1991; Talbott and Ray, 1992; Carpita and Gibeaut, 1993; Ha et al., 1997). Xyloglucan is described to be hydrogen bound to the surface of cellulose microfibrils (Keegstra et al., 1973; Hayashi et al., 1987; Bootten et al., 2004). The release of xyloglucan by cellulase activity suggested even that part of the xyloglucans are incorporated into the microfibrils without being covalently bound to cellulose (Pauly et al., 1999a). Other hemicelluloses than xyloglucan are not present in the cell wall model of dicotyledons (McCann and Roberts, 1991). Those other hemicelluloses often function as storage polysaccharides in dicotyledons.

An early model of the cell wall described a covalent linkage between pectins and xyloglucans through the reducing end of xyloglucan (Keegstra et al., 1973). This model had to be adjusted, because it is possible to extract part of the pectins from the cell wall with non-degradative solutions. Therefore, different models show the cell wall as two independent networks: the xyloglucan-cellulose network, which is embedded in a pectin network without being covalently linked to it (McCann and Roberts, 1991; Talbott and Ray, 1992; Carpita and Gibeaut, 1993).
Still circumstantial evidence is presented in literature that favours the covalent linkage: co-elution of a complex of pectin with xyloglucan on anion exchange chromatography or cellulose binding of pectin in the presence of xyloglucan (Thompson and Fry, 2000; Mort, 2002; Popper and Fry, 2005). A covalent cross link could explain the presence of pectins in the hemicellulose fraction or the residue after extraction. Such a cross link, however, cannot link all pectin molecules to the xyloglucan. Otherwise pectin would not be extractable with non-degradative solutions. Only a small number of covalent linkages within the plant cell wall would have a big effect on the strength and stability of the primary cell wall of plants. Different methods have been used with the aim to isolate an oligomer containing a pectic and a xyloglucan part (Mort, 2002). We isolated a negatively charged fraction that contained pectic galacturonic acid and xyloglucan by anion exchange chromatography. This fraction was incubated with different enzymes such as polygalacturonase, galactanase, arabinase, or rhamnogalacturonan hydrolase and afterwards reapplied on the same column. However, we were not able to show differences in elution pattern with or without enzyme incubation. Therefore, we concluded that if this cross link exists between the neutral galactan side chains of rhamnogalacturonan I and xyloglucan, the side chains are either highly branched or very short and, therefore, not degradable by the enzymes used. A second approach was to label the pectin and xyloglucan containing fraction with a fluorescent label, incubate with XEG and check for labelled xyloglucan oligomers formed on CE. However, no labelled xyloglucan oligomers were detected. The reducing ends of xyloglucan might be linked to other components, maybe pectins. Also possible is that the terminal residues are present in their reduced alditol form (see chapter 5), or that the labelled reducing ends are present in amounts lower than the detection threshold. The approach was tested by a treatment with endo-xylanase, which yielded labelled xylan oligomers. Thus, an alternative model of the cell wall could describe a pectin network that has a few connection points with the cellulose-xyloglucan network.

Another aspect we know hardly anything about is the functionality of xyloglucan side chains, although their structure is very well described (Hoffman et al., 2005). But why does the U-side chain exist? In most dicotyledons xyloglucan carry only galactose and galactose-fucose side chains (Hoffman et al., 2005). In olives an arabinose (S-side chain) replaces the fucose-galactose-unit (F-side chain) (Vierhuis et al., 2001). In contrast, bilberries and the argan tree have an additional side chain with the U-unit, which is present next to the L- and F-units. Why do members of the Ericales order need an additional side chain in xyloglucan? More work is necessary in the future to get an insight into the biofunctionality of xyloglucan side chains.
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**Effect of juice processing on cell wall polysaccharides**

**Conventional process including enzyme treatment**

Chapter 2 and 3 of this thesis describe also the analysis of polysaccharides present in juice and press cake after conventional juice processing including enzyme treatment. During conventional juice processing, cell wall polysaccharides were degraded due to the use of enzymes. In black currants, 48% of the cell wall polysaccharides present were degraded during processing. This was more than in bilberries, which contain less pectin. Only 18% of the cell wall polysaccharides present in bilberries were degraded during processing. In both berries only 6% of the cell wall polysaccharides of the berries were soluble as polymers in the juice. Approximately 46% of the cell wall polysaccharides present in black currants and approximately 76% of the polysaccharides present in bilberries remained in the press cake. The polysaccharides in the juices of black currants and bilberries showed the typical composition of modified hairy regions with high contents of rhamnose, arabinose, and galactose residues, which are residues of rhamnogalacturonan I and its neutral side chains. In addition, one third of the polysaccharides in juice were RG II, representing approximately 60% of the RG II present in the cell wall material of the berries.

The polysaccharides that remain in the press cake have a composition comparable to the composition of the residue after sequential extraction. The polysaccharides present in the seeds are the main polysaccharides present in the press cake or the residue. Next to cellulose and hemicelluloses also a remarkable amount of pectins was present. The press cakes contained 40% of the berry RG II as a dimer incorporated into the pectin network. It was released by the use of polygalacturonase combined with pectin methyl esterase.

**High pressure processing**

The effect of high pressure processing on cell wall polysaccharides is described in chapter 6. We showed that pectins present in berry mashes contained less methyl esters after high pressure processing of the mashes. The deesterification of pectins is probably not an independent chemical reaction, but catalysed by the enzyme pectin methyl esterase, which is endogenously present in berries. An explanation for the decrease of methyl esterification is a possible activation of endogenous pectin methyl esterase, as described before for other fruits (Hernandez and Cano, 1998; Krebbers et al., 2003). High pressure is able to change the tertiary structure of the enzymes, which can influence their activity (Balny and Masson, 1993; Hendrickx et al., 1998).

In addition, pectins became more extractable after high pressure processing. Less galacturonic acid residues were left in the fraction extracted with diluted alkali solution and in the hemicellulose fraction. More galacturonic acid residues were found in the fraction extracted with hot buffer containing chelating agent (bilberries) or the fraction extracted with hot buffer (black currants). A change in the degree of methyl esterification of pectins was shown to occur only in the calcium sensitive fraction of both berries. Under high pressure processing a
specific pectic population was modified, which was not the easiest accessible population. Probably pectin methyl esterases present close to these pectic populations are responsible for this effect. It is known that polysaccharide modifying enzyme, especially pectin methyl esterases, is present in specific domains of the cell wall (Benen et al., 2003). After mashing, the enzymes remain in those domains and can only act on the pectins present in those domains. The strong binding of pectin methyl esterase to specific cell wall domains is indicated by the extraction procedure of the enzyme: to extract pectin methyl esterase a high ionic strength and preferably a surfactant are necessary to release the enzyme from the cell wall material (Versteeg et al., 1978; Barnavon et al., 2001). Another possibility is that the degree of esterification or the degree of blockiness influence the binding of the enzyme to its substrate (Daas et al., 1999). The active enzyme is only able to bind to pectins which have a certain pattern of methyl esterification. It cannot be excluded that there are several events happening in the berry mash and that not only the degree of methyl esterification is responsible for the extractability. Maybe the extractability of pectins is influenced by other effects, physical or biochemical.

After high pressure processing pectins became more calcium sensitive due to a decrease in their degree of methyl esterification and became easier extractable. These findings together explained the formation of a stronger pectin gel after high pressure processing of bilberry and black currant mash. As a consequence of this stronger gel, the viscosities of the mashes were increasing and pressing became more difficult (Poutanen and Kara, 2006).

Combined High pressure and enzyme treatment

After analysing the effect of high pressure processing on cell wall polysaccharides, the processing was combined with enzymatic treatment and the results are described in chapter 6, as well. To study the effect of commercial enzymes together with high pressure processing, one hemicellulolytic and two pectolytic enzyme preparations were added to three different berry mashes directly before high pressure was applied on the samples. After pressurisation at 400 MPa for 15 min, the mash was left at room temperature for two hours to enable optimal enzymatic degradation. Afterwards the composition of cell wall polysaccharides was determined in the berry mash and changes in activity of different enzymes under high pressure were extrapolated from the obtained results.

Pectic polysaccharides were further degraded if enzymes were added and high pressure was applied. At atmospheric pressure, contents of galacturonic acid residues decreased in the polymeric material after addition of the different pectolytic enzymes. The same accounted for galactose, arabinose, and rhamnose residues. After treatment with high pressure the contents of galacturonic acid and rhamnose residues decreased even further, while the contents of galactose and arabinose residues decreased to a similar extent as under atmospheric pressure. Probably polygalacturonase and pectin methyl esterase were not only pressure stable, their activity on homogalacturonan was even enhanced when
pressure was applied. Polygalacturonase itself need not necessarily to be activated to give the observed effect. An enhanced activity of pectin methyl esterase would produce more substrate for polygalacturonase. This would lead to a further decrease of the galacturonic acid content in the polymeric material after high pressure processing. However, if fungal pectin methyl esterase is activated similar to plant pectin methyl esterase, has not been investigated in isolated model system. It can also be speculated that a polygalacturonase-homogalacturonan-complex is formed under the applied pressure, which enhances the activity of polygalacturonase. In this case the activity of polygalacturonase remains the same before and after high pressure processing. This effect can only be investigated when the enzymes are pressurised in a food matrix.

Rhamnogalacturonan I was further degraded if high pressure was applied. Probably rhamnogalacturonan hydrolase or rhamnogalacturonan lyase were activated under high pressure. Another possibility is that a complex of rhamnogalacturonan I and its degrading enzymes is formed under high pressure (similar to the polygalacturonase-homogalacturonan described earlier). An important aspect is that the content of rhamnose decreases in the polymeric fraction. Rhamnogalacturonan I was not only degraded, but the rhamnose residues were released as small oligomers so that they were removed from the mash by washing with 70% alcohol solution. It is possible that rhamnogalacturonan I degrading enzymes could access their substrate more easily due to the removal of the arabinan and arabinogalactan side chains from the backbone.

Hemicelluloses were only degraded when treated with enzymes under atmospheric pressure. The contents of the hemicellulolytic sugar residues mannose, xylose, and glucose decreased when the mash was treated with enzymes under atmospheric pressure, but when pressure was applied these decreases were hardly observable. The pressure of 400 MPa seems to inactivate the hemicellulases so that they were not able to degrade hemicelluloses anymore.

**Consequences for producers of enzyme preparations**

The juice processing industry has developed to a modern biotechnology industry. As processing aids, different pectolytic enzyme preparations are on the market. However, development of these enzyme preparations was often based on trial and error. The detailed characterisation of the cell wall polysaccharides of bilberries and black currants, which is described in this thesis, is a basis for the development of enzyme preparations that improve juice processing. Pectolytic enzymes are more effective in black currant processing, because black currants contain more pectins than bilberries as described in chapter 2. The degree of methyl esterification has to be taken into account, as well. In bilberries, which have a high degree of methyl esterification, activity of pectin methyl esterase or pectin lyase is more important than in black currants, which have a lower degree of methyl esterification.
Other aspects are the neutral side chains of the pectic macromolecules. They can mainly be found in the pectins that are difficult to extract, probably because they can hydrogen bind to cellulose (Zykwinska et al., 2005; Zykwin ska et al., 2006). Degradation of these side chains or the rhamnogalacturonan I backbone could release more homogalacturonan, which is then easier accessible to degradation by polygalacturonase in combination with pectin methyl esterase or pectin lyase. An enzyme can easier bind to a soluble substrate. But are rhamnogalacturonases, galactanases, or arabinases pectolytic enzymes? The commission directive 96/77/EC defines pectins as containing not less than 65 % galacturonic acid (96/77/EC, 2004). By this definition, rhamnogalacturonan and its neutral side chains are part of pectin. Consequently rhamnogalacturonases, galactanases, and arabinases are pectolytic enzymes, because they are able to degrade part of the pectin molecule. In bilberries and black currants, degradation of rhamnogalacturonan I and the neutral side chains is important to improve the accessibility of the tightly bond pectin for an optimal degradation. As part of arabinogalactan proteins, the neutral sugar side chains might be responsible for the binding of pectin to the cell membrane. Arabinogalactan proteins have a membrane anchor (Youl et al., 1998) and are probably covalently linked to pectins (Morvan et al., 2003; Immerzeel, 2005). Arabinogalactan proteins contain side chains of arabinogalactan type II (1,3/1,6-linked galactan backbone) (Schols and Voragen, 2002). Pectic arabinogalactan has mainly a 1,4-linked galactan backbone (type I) (Schols and Voragen, 2002). The more difficultly extractable pectins of bilberries and black currants are, the higher is the content of type II arabinogalactan (chapter 2).

Rhamnogalacturonan II (RG II) was shown to play an important role as a cross linker of pectins in the cell walls (chapter 3). What happens if rhamnogalacturonan II is enzymatically degraded, e.g. unbranched or degraded in the backbone? A removal of single sugar residues in an exo-way is not expected to have a big pact. To date there is no enzyme preparation that is able to degrade rhamnogalacturonan II on the market. Although RG II degrading activity was found in *Penicillium daleae* (Vidal et al., 1999), no food grade enzyme preparation could be produced. On the one hand specific degradation of rhamnogalacturonan II could destroy the pectin network. This could lead to novel products containing polymeric pectin fragments. These polymeric fragments can function as dietary fibre or as endogenous gelling agent, e.g. for jams, but they can make processing more difficult due to increased viscosity of the product. On the other hand, RG II degradation in combination with existing pectolytic enzyme preparations could lead to a more extensive degradation of pectins. In current processing 40 % of RG II remain in the press cake incorporated in pectic polysaccharides. Degradation of these RG II cross links might result in a better accessibility of the pectins covalently linked to RG II dimers. This would results in higher juice yields and probably in higher contents of soluble polyphenols in the juice.

It could also be interesting to release more intact RG II from the cell wall material into the juice. RG II is able to bind heavy metal ions (Matsunaga and
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Ishii, 2004). These ions are toxic in their free form, but when complexed by RG II these ions are not bioavailable any more. Such an effect is considered as health promoting and is desirable.

**Consequences for the berry processing industry**

Juice yield and content of polyphenols in the juice have primary interest of the berry processing industry. Polyphenols are responsible for the colour and colour is one criterion for the price of the juice. Furthermore, polyphenols are discussed to have health promoting activity. The content of soluble dietary fibre in the juice has potential for the health promoting function of the juice, as well (Anderson, 1990; Yamada, 1994; Sembries et al., 2003; Sembries et al., 2004). Increase of the dietary fibre is a challenge for the enzyme producers. They have to restrict the enzyme activities with the aim of keeping polymeric cell wall material in the juice, but without increasing the viscosity too much and, therefore, raising technological problems. Haze formation, which is of minor interest in berry juice due to the intensive colour of the juice, might become more problematic when high amounts of polysaccharide are present. More easy to obtain are non-digestible oligosaccharides, some of which are discussed to have prebiotic effects (Palframan et al., 2003). Again, producers of enzyme preparations have to adapt their products not to degrade pectic oligosaccharides into monomers. If these oligomers can be obtained in physiological active quantities, has to be investigated.

Highest potential for improving juice yield and quality have novel and tailor made enzyme preparation as described before. Recent investigations on combing different enzymes by sequential addition to the substrate showed synergistic effects (Sorensen et al., 2006). It is worthwhile to test a combination of complementary commercial enzyme preparation in a one-step or successive way.

The development of monoclonal enzymes is another prospering field. These enzymes would enable tailor made enzyme preparations. Novel physical treatments such as high pressure processing can have synergistic effects together with enzyme treatments. However, as long as high pressure can only be applied batch wise, it remains an expensive treatment. This treatment is only commercially relevant in high quality products and is applied only after packaging of the final product for pasteurisation purposes. Broad application of high pressure processing can be an improvement of berry processing, but continuously working units are necessary for commercial relevance. High pressure processing is changing the texture of a berry mash. Other applications for improved texture of fruit products are also possible in the future.

Next to improvement of juice yield and quality in berry processing, novel products obtained from the press cake become more and more important. These co-products can be obtained by enzymatic treatment of press cakes with the aim of producing dietary fibre, polyphenolic extracts, or even bioethanol. The obtained products have to have a much higher value than the press cake, which is currently burned to obtain energy.
Results of the MAXFUN project

Within the project ‘Novel enzyme-aided extraction technologies for maximised yield and functionality of bioactive components, products, and ingredients from by-products’, acronym MAXFUN, the berry and grape processing habits were revealed (Poutanen and Kara, 2006). In a first approach commercial enzyme preparations were characterised and tested for their effect on juice yield and juice composition (Buchert et al., 2005). The effect of enzyme preparations on juice yield and total phenolic concentration in the juice was shown. With all enzymes used the juice yields and the concentrations of polyphenols in the juice were significantly increased. However, this approach is a model study. The enzyme dosages were much higher than in the commercial process. In our study on the effect of high pressure treatment in combination with high pressure processing, we chose enzyme dosages related to industrial processes (chapter 6). For the determination of enzyme activities the pH of the working environment is important. Black currant mash has a pH of 2.6 and bilberry mash of 2.7, respectively. The characterisation of commercial enzyme preparations was performed at pH 3.5, which can provide indications for activities at the pH of berries, but even major differences in activity between pH 3.5 and pH 2.7 are possible.

Polyphenols were also enzymatically changed during processing. Glycosyl residues could be split off resulting in improved bioavailability (Gonzalez-Barrio et al., 2004), but in some cases also in decreased stability (Poutanen and Kara, 2006).

In another approach a novel fungus able to degrade rhamnogalacturonan II was identified. Although the enzyme (mixture) could not be purified, this fungus offers the opportunity to obtain RG II degrading enzymes in commercial scale in the future (Trindade et al., 2006).

The MAXFUN-project broadened the knowledge on berry processing. Next to the detailed characterisation of berries described in this thesis, important progress has been made on the development of novel enzymes preparations and polyphenol analysis in the juice and berries. Although translation to industrial scales has not always been performed, the results of the project show new pathways and possibilities to improve juice processing in the future.
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Summary

Samenvatting

Zusammenfassung
Summary

Bilberries and black currants are important crops in northern Europe due to their strong colour and attractive flavour. Most of the berries are processed to juice, which can be diluted and consumed as nectar or used as a food ingredient. Cell wall polysaccharides play an important role during berry processing. Due to their polymeric character they can cause problems during clarification or cause thickening of the mash after homogenisation, which leads to lower juice yields. Commercial enzyme preparations are used to degrade cell wall polysaccharides during processing. However, the cell wall composition of berries has never been studied in detail.

To increase juice yield and functionality of the juices, especially enzymatic, but also physical treatments were investigated in the framework of an EC-project. Monitoring of the affected components or a tailored degradation was needed to optimise juice processing. Aim of this study was to determine the structure and composition of cell wall components in berries and to use the obtained data to analyse the effect of conventional and high pressure processing on cell wall polysaccharides.

Chapter 1 gives a general introduction to bilberries and black currants including their possible health effects. The current processing is described, during which pectolytic enzymes are used to degrade the plant cell walls. Therefore, an overview about structural elements and architecture of the plant cell wall is given, as well.

A general characterisation of cell wall polysaccharides in bilberries and black currants was performed of the whole berries and of the different tissues, viz. skin, pulp, and seeds (chapter 2). Black currants contained more pectin than bilberries, while the degree of methyl esterification was lower in black currants. The seeds of berries are dominated by storage polysaccharides, which are hemicelluloses. The major storage polysaccharides were mannans in black currant seeds and xylans in bilberry seeds.

In chapter 3 the role of rhamnogalacturonan II, the most complex structural element of pectins known to date, was investigated in berries. Rhamnogalacturonan II was quantified in all pectic fractions of bilberries and black currants via its diagnostic sugars 2-O-methyl fucose and 2-O-methyl xylose. Rhamnogalacturonan II represents between 5 and 10% of the pectins and was released from the polymeric pectins by polygalacturonase, which is an enzyme able to degrade demethylated homogalacturonan. Rhamnogalacturonan II was released in the dimeric form, esterified via boric acid. This supports the theory that rhamnogalacturonan II functions as a cross link of two pectin chains in the cell wall.

While the storage hemicelluloses of the berry seeds were not further characterised than described in chapter 2, xyloglucans of black currants and bilberries were analysed in the cell wall material or after partial isolation. The xyloglucans were degraded using xyloglucan specific endo-glucanase and the
obtained oligomers, typical building blocks of the polymer, were analysed using different chromatographic, mass spectrometric, and capillary electrophoretic techniques. Using black currant xyloglucan as an example, the different techniques were compared in chapter 4. Xyloglucans of black currants had a rather simple structure with XXXG-building blocks and fucose and galactose containing side chains (L and F). The galactose residues of both L- and F-side chains were partly esterified with acetyl groups. When bilberries were analysed for their xyloglucan structure, major differences compared to black currants were observed (chapter 5). Bilberry xyloglucans had a complex structure with more than 20 different building blocks. The main structure was XXXG-type structure, but some XXG-type oligomers were present, especially when the oligosaccharides were derived directly from the cell wall material. Some of these building blocks contain beta-linked xylose in the side chains, which was designated as U-side chain. U-side chains have been described before for the argan tree, which is together with bilberries a member of the Ericales order. Four blocks that were identified in bilberries have not been described in literature.

During conventional processing, which includes an enzyme treatment, pectic polysaccharides were degraded (chapter 2). This degradation was more extensive in black currants than in bilberries due to the higher pectin content. A small amount of the polysaccharides was also extracted into the juice in polymeric form. One third of these juice polysaccharides were rhamnogalacturonan II in its monomeric and dimeric form (chapter 3). The polysaccharides in the press cake were dominated by polysaccharides originating from the seeds. Therefore, the main sugar residues present in the polymeric material were mannose in black currants and xylose in bilberries. The press cakes contained still a high amount of pectins, which originate only partly from the seeds.

After conventional juice processing, changes in cell wall composition during high pressure processing were monitored (chapter 6). Pectins in berry mash become more calcium sensitive and more extractable after treatment with high pressure. This leads to a decrease in juice yield. When high pressure processing is combined with a treatment using commercial enzyme preparations, pectins are further degraded than at atmospheric pressure (chapter 6). Polysaccharides containing galacturonic acid and rhamnose residues were further degraded to mono- and oligomers when enzymes were applied at high pressure. Hemicellulases are, however, inactivated by the pressure studied here. The level of hemicellulolytic residues xylose, mannose, and glucose decreased in the polymeric material when treated with commercial enzyme preparations at atmospheric pressure, but remained almost the same when treated with the preparations at high pressure.

In chapter 7 the achievements of the previous chapters are summarised and discussed in the context of plant cell wall architecture. Furthermore, consequences for producers of enzyme preparations and for the berry processing industry are discussed.

The detailed characterisation of cell wall polysaccharides in bilberries and black currants lead to the identification of novel polysaccharide structures and
showed the complexity of plant cell walls. Influences of novel processing technologies were measured. A higher viscosity and lower juice yield after high pressure processing were assigned to changes in pectin structure and extractability. Based on this knowledge, a synergistic effect was obtained by combining high pressure processing with treatment with commercial enzyme preparations.
Samenvatting

Bosbessen en zwarte bessen zijn vanwege hun donkere kleur en sterke aroma commercieel belangrijke vruchten in Noordeuropa. De meeste bessen worden verwerkt tot sap, dat verdund als nectar gedronken kan worden of als ingrediënt wordt gebruikt. Celwand polysacchariden spelen een belangrijke rol tijdens het verwerken van bessen. Vanwege hun polymeer karakter kunnen ze tijdens het filtreren problemen veroorzaken of ze kunnen de bessenpuree verdikken, wat een reden is voor lagere sapopbrengst. Tijdens de verwerking worden commerciële enzym preparaten aan de bessen toegevoegd om de celwandpolysacchariden af te breken. De samenstelling van celwand polysacchariden in bosbessen en zwarte bessen is tot op heden nog niet gedetailleerd geanalyseerd.

Om de opbrengst en functionaliteit van het sap te verbeteren, werden binnen het kader van het EU-project MAXFUN enzymatische en fysische behandelingsmethoden onderzocht. Het herkennen van veranderingen of substraatspecifieke afbraak van celwandcomponenten is nodig, om de sapproduktie te optimaliseren. Het doel van dit proefschrift was de structuur en samenstelling van celwanden uit bosbessen en zwarte bessen te bepalen en de resultaten te gebruiken om de effecten van conventionele verwerking en hoge druk behandeling op de chemische structuur van celwandpolysacchariden te bepalen.

Hoofdstuk 1 geeft een introductie over bosbessen en zwarte bessen inclusief hun mogelijk gezondheidsondersteunende eigenschappen. Het conventionele sapproductie proces wordt beschreven, waarbij commerciële enzym preparaten gebruikt worden om celwanden af te breken. Om de werkwijze van enzymen te verduidelijken is een overzicht over de opbouw en samenstelling van de celwand gegeven.

Celwandpolysacchariden in bosbessen en zwarte bessen werden gekarakteriseerd in hele bessen, schil, vruchtvlees en pitten (hoofdstuk 2). Zwarte bessen bevatten meer pectine dan bosbessen, maar de methanolveresteringsgraad is lager in zwarte bessen. De pitten van bessen bestaan vooral uit hemicellulosen, aanwezig als opslag polysacchariden. Het belangrijkste opslagpolysacchariden in zwarte bessen is mannaan en xylaan in bosbessen.

In hoofdstuk 3 wordt de functie van het complexe structuurelement rhamnogalacturonaaan II beschreven. Rhamnogalacturonaaan II werd gekwantificeerd in alle pectinefracties van bosbessen en zwarte bessen met behulp van de diagnostische suikers 2-O-methyl xyloose en 2-O-methyl fucose. Rhamnogalacturonaaan II heeft een aandeel van 5 tot 10 % in pectine en wordt vrijgemaakt uit polymeer pectine met polygalacturonase. Het vrijgemaakte rhamnogalacturonaaan II is een dimer van twee monomeren veresterd met boorzuur. Dit resultaat bevestigt de hypothese dat rhamnogalacturonaaan II twee pectineketens in de celwand met elkaar kan verbinden.

Tijdens de conventionele bessenverwerking met enzymincubatie wordt pectine afgebroken (hoofdstuk 2). Celwanden van zwarte bessen worden verder afgebroken dan celwanden van bosbessen omdat zwarte bessen meer pectine bevatten. Een kleine hoeveelheid polysacchariden komt als polymeer in het sap. Een derde deel van deze polysacchariden is rhamnogalacturonan II mono- en dimer (hoofdstuk 3), twee derde deel is afkomstig uit de gemodificeerde vertakte gebieden (modified hairy regions). De polysacchariden in de perskoek worden gedomineerd door de opslagpolysacchariden uit de pitten. Daarom is de meest voorkomende suiker in bosbessen xylose en in zwarte bessen mannose. De perskoeken bevatten nog steeds een hoog gehalte aan pectine, voornamelijk afkomstig uit de pitten.


In hoofdstuk 7 worden de resultaten van de vorige hoofdstukken samengevat en besproken in samenhang met de celwandarchitectuur. Verder worden de
consequenties voor enzymproducenten en voor de bessenverwerkende industrie besproken.

De gedetailleerde karakterisering van celwandpolysacchariden in bosbessen en zwarte bessen leidde tot de identificatie van nieuwe polysaccharidenstructuuren en onderstreept opnieuw de complexiteit van de plantencelwand. De invloed van een nieuwe procestechnologie is ook bepaald. Een hogere viscositeit en een lagere sapopbrengst konden worden teruggekoppeld aan veranderingen in de pectine structuur. Op basis van deze kennis kon een synergistisch effect worden bereikt door de combinatie van hoge druk en enzym behandeling.
Zusammenfassung


Um höhere Saftausbeuten und eine erhöhte Funktionalität des Saftes zu erreichen, wurden insbesondere enzymatische, aber auch physikalische Behandlungsmethoden innerhalb des EC-Projektes MAXFUN untersucht. Zur Optimierung der Prozeßschritte ist es notwendig, Bestandteile der Zellwände, die durch die Prozesse beeinflusst werden, analytisch zu begleiten und deren enzymatischen Abbau gezielt zu fördern. Das Ziel dieser Doktorarbeit war es daher, die Zellwandstruktur und -zusammensetzung von Heidelbeeren und schwarzen Johannisbeeren zu ermitteln und die erhaltenen Daten dazu zu verwenden, Veränderungen an Zellwandkomponenten durch konventionelle Saftherstellung oder durch Hochdruckbehandlung zu erkennen.

Kapitel 1 gibt einen Überblick über Heidelbeeren und schwarze Johannisbeeren im Allgemeinen und deren mögliche gesundheitsfördernde Eigenschaften. Desweiteren wird in diesem Kapitel die konventionelle Beerenverarbeitung beschrieben, während der üblicherweise pektolytische Enzympräparate verwendet werden, die die durch Pektine geformten gelartigen Strukturen abbauen und so die Zellwandstrukturen teilweise zerstören. Um diesen Effekt zu verdeutlichen, ist in diesem Kapitel ebenso ein Überblick über die Strukturelemente und den Aufbau der Zellwände enthalten.


Neben dem konventionellen Prozeß wurden auch Veränderungen in der Zellwandzusammenstellung während einer Hochdruckbehandlung untersucht (Kapitel 6). Durch Hochdruckbehandlung bekommen Pektine eine höhere Calciumaffinität und werden leichter extrahierbar. Dieses führt zu verminderten

In Kapitel 7 werden die in den vorherigen Kapiteln erhaltenen Resultate und Ergebnisse zusammengefasst und im Kontext von Zellwandaufbau und prozeßbedingten Veränderungen diskutiert. Desweiteren werden Konsequenzen für Hersteller von Enzympräparaten und für die Beeren verarbeitende Industrie erörtert.

Acknowledgement

“Do you really want to go to Holland for four years?” was what I heard quite often after I decided to start my PhD studies here in Wageningen. I went and it was the best decision I could make. I was very warmly welcomed here in the Lab and everywhere else like at my football club SKV or at the orchestra De Ontzetting. I experienced the Dutch as very friendly, open, and direct people and I enjoyed living with them. Wageningen University is, in my opinion, a frontrunner. Bologna is common practice here and research is performed on a high level. I hope that I was able to contribute to this with my thesis you are reading here. Of course such a thesis would not have been possible without the help and support of many people.

First of all I like to thank my promotor Fons Voragen and my co-promotor Henk Schols. I am very glad that you accepted me as a PhD student. From the beginning on we had a lot of in-depth scientific discussions and you helped me to understand the plant cell wall and its components as far as it is known to date. And we know that there is still so much to learn about plant cell walls. Next to science itself we were also discussing “politics” in science. It was always very interesting. Thank you very much!

Furthermore, I want to thank Fons Voragen and Harry Gruppen for the research condition I found in the Laboratory of Food Chemistry. I was really surprised to find the laboratory very well equipped and all equipments running for most of the time. You both run a food chemistry laboratory which is quite unique in the world, I would say. Keep it that way!

Seven students contributed with their MSc or BSc theses to the work described in this booklet. Although not all of your work has been published in scientific articles, all of you made an important contribution to the content of this thesis and to the contents of the MAXFUN reports. Thank you very much Femke, Lies, Tanya, Xiaowei, Laura, Martine, and Maarten. All the best for your future!

From the MAXFUN-team I learned a lot about the science of grapes and berries and also about EC-projects during the last four years. Many thanks to Kaisa Poutanen and Mirja Mokkila for coordinating the project! I enjoyed every meeting with Johanna, Martina, Pasi, Annikka, Paco, Juan Carlos, Rocio, Leo, Luisa, Veronique, Jean-Marc, Cecile, Riita, Anja, Jani, Marisa, David, Vernu, Steffen, Keld, Saska, and Heiki. Next to science we enjoyed nice dinners and sometimes a beer in Wageningen, Helsinki, Murcia, Kuopio, or Montpellier. Thank you! I want to thank also Thierry, Pascale, and Anisia for welcoming me in Montpellier for two weeks, which resulted in chapter 3 of this thesis.

There are some people who made life much easier for us PhD students in the lab: the technicians who take care about our equipment and always try to help with specific research questions. Thank you Edwin, Jan, Margaret, Ben, René, Mark Sanders, Toos, Jolan, and Aagje! You saved me a lot of time!

Another group of people who helped me a lot not only to start up very efficiently, but continuously throughout the four years was the polysaccharide
group. We had very interesting discussions about science and also other things, sometimes with a beer. Such a group of experts on polysaccharides was a big advantage on formulating a scientific hypothesis and on troubleshooting during analytical work. Thank you René, Mirjam, Stéphanie Guillotin, Gerd Jan, Joris, Edwin, Daan, Mark Dignum, Bram, Sandra, Yvonne, Junrong, Tai, Peter Immerzeel, Jean-Paul, Gerrit Beldman, and Chen!

I could also discuss with all “non-polysaccharide” people in the lab often about your topics and analytical techniques, but also politics and other things. I like to thank Bas, Wil, Koen, Maaike, Gerrit van Koningsveld, Nathalie, Julia, Karel, Peter Wierenga, Kerensa, Walter, Johan, Jacques, and Sergio. A special thanks goes to all my room mates starting with Edwin in 527, Laurice, AnneMarie, Evelien, Stéphanie Prigent, Karin, Koen, and Lidwien in 509, and afterwards Gerd Jan, Stephanie Guillotin, and Edwin in 517. I always enjoyed working in your company.

There is one person in lab that helped me with all the organisatorical things, which are quite a lot during four years of work and a number of congresses visited. You even took good care that me and Fons did not forget our appointments! Thank you Jolanda!

I would like to say thank you to Prof. Steinhard, Dr. Paschke, and all the other people at the Department of Food Chemistry, Hamburg University, who prepared me for this PhD thesis here. The education I got was quite an advantage in the last four years. Especially the time we spent working independently in the laboratory during our studies was very valuable.

You probably saw that I did not design the cover myself. Due to my underdeveloped artistic skills I asked professional help to make a cover with berries. Mark, you gave my thesis the cover it deserved. Thank you very much, it looks great!

I like to thank Karin, Bas, and Gerd Jan for the nice time we spent on organising the study trip to Japan. Although I had to get used to lunch meetings, they were always nice and became a habit, which I missed afterwards on Wednesdays. We managed to bring 24 PhD students and 3 professors safely to Japan and back full of new scientific and cultural impressions. The organisation was exciting and interesting and thanks to Rob we managed the financial accountance without any problem. I would like to take the opportunity to thank Evelien, Koen, Maaike, Cathrine, and Neleke for organising the trip to Belgium, France, and the UK in 2006. I know how much work it is and I enjoyed the trip a lot.

A special thank you goes to my two paranymphs Gerd Jan and Wil and also to Bas. Thank you for your scientific opinion, your friendship, and all the football matches we enjoyed in front of a television or in the stadium. Bennekom against Willem II was much more exciting than Ajax against Espanyol!
Acknowledgement

I would also like to thank my parents, my sister Amrei, and her fiancé Kenneth. Ohne Eure Unterstützung in kleinen und in großen Fragen wäre ich gar nicht in der Lage gewesen, zu studieren, geschweige denn, eine Doktorarbeit im Ausland zu schreiben! Vielen Dank!

Am Ende dieser Arbeit möchte ich mich bei Dir, Yee, ganz herzlich für Deine Liebe und Unterstützung, nicht nur während der letzten vier Jahre bedanken!
Curriculum vitae

Hauke Hilz was born on the 29th of May 1977 in Stade, Germany. He grew up in Harsefeld, Germany, and finished school at the Gymnasium Athenaeum Stade in June 1996. In April 1997 he started his studies in Food Chemistry at the Hamburg University after 10 months of military service. Hauke graduated under Prof. Dr. Dr. Steinhart with his 1st state examination, which included the diploma in Food Chemistry in September 2001. His diploma thesis was entitled ‘Studies concerning the antioxidative capacity of melanoidins in roasted coffee’. Afterwards Hauke started his professional training for one year, which he finished with the 2nd state examination in December 2002 to become a certified Food Chemist. During this year he did an internship at Unilever Research Vlaardingen, The Netherlands, on HPLC determination of polyphenols in olive oil and another internship at the Hygiene Institute Hamburg, Germany, on regulation, analysis, and legal assessment on food stuff, cosmetics, and related products in a governmental food inspection laboratory. The research on cell wall polysaccharides of bilberries and black currants described within this thesis was carried out at the Laboratory of Food Chemistry, Wageningen University, from January 2003 until December 2006.
List of publications

Full publications

Abstract publications
Overview of completed training activities

**Discipline specific activities**

**Courses**
Glycoscience Summer School, Wageningen, 2004
Visit to INRA Montpellier, France, 2005

**Meetings**
MAXFUN Meetings, Europe, 2003-2005
Food Chemistry Seminar, Wageningen, 2003-2006
32nd German Food Chemists Day, Munich, Germany, 2003
COST D29: Production and Functionalisation of Hemicelluloses for Sustainable Advanced Products, Bergedorf, Germany, and Wageningen, 2004
Food Chemistry Symposium, Hamburg University, Hamburg, Germany, 2004
33rd German Food Chemists Day, Bonn, Germany, 2004
X. Cell Wall Meeting, Sorrento, Italy, 2004
44th VLAG Meeting: Enzymatic Conversion of Biopolymers, Wageningen, 2005
EFFoST Congress Processing Developments for Liquids, Cologne, Germany, 2006
Gordon Research Conference Plant Cell Walls, Biddeford, Maine, USA, 2006
3rd Conference on Biocatalysis in the Food and Drinks Industries, Wageningen, 2006

**General Courses**
Scientific Writing, Wageningen, 2003
VLAG PhD Introduction Days, Bilthoven, The Netherlands, 2003
Introduction to Management and Marketing, Wageningen, 2003
Course on Preparation for Performance and Development Evaluation, Wageningen, 2004
Philosophy and Ethics of Food Science and Technology, Wageningen, 2006
Leadership for Young Professionals, Düsseldorf, Germany, 2006

**Optionals**
Student Presentations at the Laboratory of Food Chemistry, Wageningen, 2003-2006
PhD Trip Food Chemistry to Japan as Member of the Organising Committee, 2004
PhD Trip Food Chemistry to Belgium/France/United Kingdom, 2006
The research described in this thesis was performed at the Laboratory of Food Chemistry, Wageningen University, The Netherlands. This study has been carried out with financial support from the Commission of the European Communities, specific RTD program ‘Quality of Life and management of Living Resources’, contract number QLK1-CT-2002-02364 ‘Novel enzyme-aided extraction technologies for maximized yield and functionality of bioactive components in consumer products and ingredients from by-products’, acronym MAXFUN. It does not necessarily reflect its views and in no way anticipates the Commission’s future policy in this area.