



A novel perspective on pectin extraction

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A novel perspective on pectin extraction



Malgorzata Maria Dominiak

Ph.D. Thesis

2014

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Preface

This thesis is based on work carried out between August 2010 and December 2013 at DuPont Nutrition Biosciences ApS, Department of Hydrocolloid Science under the supervision of Principal Scientist Karen Marie Søndergaard, and at the Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering, Technical University of Denmark under the supervision of Professor Jørn Dalgaard Mikkelsen.

The PhD study was a part of the Lean Green Food project (EU-ITN 238084) and was financed by DuPont Nutrition Biosciences ApS, Technical University of Denmark and Marie Curie 7th Framework Program.

I would like to thank my supervisor Jørn Dalgaard Mikkelsen for inspiration, encouragement and valuable discussions. My co-supervisor, Karen Marie Søndergaard, is thanked for help, support and valuable comments. Anne Grete Juul from the Department of Hydrocolloid Science, DuPont Nutrition Biosciences ApS, is acknowledged for her help with project organization and troubleshooting.

Grit Fischer, Maren Plewe and Constanze Doese from DuPont Germany are acknowledged for their hospitality and excellent company during my research stay in Niebüll, Germany.

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Finally I would like to thank my family, especially my husband Dominik, for always being there for me.

Malgorzata Maria Dominiak

DTU, 13th of January, 2014

This thesis is dedicated to the memory of my father, Andrzej Sniady.
I am glad he saw this process, offering me support and encouragement.

Abstract

The classical (current) extraction of pectin is based on an acid-catalyzed process, using nitric, hydrochloric or sulphuric acid. The reaction takes place at low pH and high temperatures for several hours. The main disadvantage of this technology, and one which raises environmental concerns, is generation of large volumes of acidic effluent, which require further treatment before release. The main focus of this PhD study was to replace acid with enzymes and thereby achieve sustainable, green production of pectin. The first goal was to prove that an enzyme-based process could generate pectin with the same yields and functional properties as an acid-based process. 13 commercial enzymes were selected for the primary screen and 4 were examined in larger scale extractions. The best enzyme, Laminex C2K, gave a yield of 23%, had a molecular weight of 69 kDa, and possessed functional properties comparable with pectins obtained in a classical way. In the future it would be beneficial to optimize the Laminex C2K production strain (*Penicillium funiculosum*) by molecular design to delete the residual pectinolytic activity and include plant cell wall hydrolases.

Pectin production is complex and therefore its optimization is a long process because the evaluation of the final product quality is accomplished at the end of the procedure, employing time-consuming off-line laboratory tests. Fourier transform infrared spectroscopy (FTIR) and carbohydrate microarrays, combined with chemometrics, were evaluated for their abilities to predict of pectin yields and assess pectin traits during the pectin extraction process. Using crude lime peel extracts, both FTIR and carbohydrate microarray analysis showed predictive and descriptive abilities with respect to acid and enzymatically extracted pectins. Furthermore, FTIR determined the optimal extraction time for both the enzymatic and acidic extraction processes. The combined results suggested major differences in the crude pectin extract traits of enzymatically vs. acidically extracted pectin with respect to the degree of esterification, purity, and abundance of rhamnogalacturonan I pectic regions.

Although the major pectin applications still include gelling, thickening and stabilization, other novel uses employ its prebiotic potential, anti-cancer properties and heavy metal detoxification ability (Hotchkiss, Rastall, Gibson, Eliaz, Liu, & Fishman, 2009). Sunflower pectin was extracted and fractionated into three fractions according to size, SPF<50, SPF50-100 and SPF>100, using 50 and 100 kDa membranes. The density of *Bifidobacterium* spp. was significantly higher ($p<0.05$) after fermentation on SPF>100 kDa. All three sunflower pectin fractions did not influence the level of *Lactobacillus* spp., *Bacteroidetes* and *Firmicutes*.

Dansk sammenfatning

Den klassiske ekstraktion af pektin er baseret på en syrekatalyseret proces, hvor der anvendes salpetersyre, saltsyre eller svovlsyre. Reaktionen sker ved høj temperatur, lavt pH samt en reaktionstid på flere timer. Ulempen ved denne proces er oprensningen af store mængder uorganiske kemikalier. Hovedfokus på dette PhD studie er at erstatte syrer med enzymer og dermed opnå en bæredygtig grøn produktion af pektin. Det første mål var at bevise, at et enzym baseret proces kunne fremstille pektin med samme udbytte og funktionelle egenskaber, som den klassiske proces. Der blev anvendt 13 forskellige kommercielle enzymer til den første screening, og 4 blev valgt til den endelige afprøvning i større skala. Det bedste enzym, Laminex C2K, gav et udbytte på 23% og en mol vægt på 69 kDa, samt en gelerings og viskositetseffekt, der var på højde med de kvaliteter af pektin man kan fremstille ved den klassiske metode.

Pektin processen er meget kompleks og optimeringen af reaktionsbetingelserne og kvaliteten af det endelige produkt bliver normalt målt i slutningen af processen med arbejdskrævende analyser. Fourier transform infrared spectroscopy (FTIR) og kulhydrat "micro-array" kombineret med chemometrics, er blevet anvendt til at bestemme pektin udbytte og bestemme pektin struktur under ekstraktions processen, ved at måle direkte på ekstraktions blandingen. Begge metoder kunne forudsige den optimale ekstraktionstid, samt måle mængden af methyl estere, niveau af urenheder og mængde af RGI.

Selv om applikationen af pektin er rettet mod gelering, fortykningsmidler eller stabilisering, er der også et stigende behov for pektin som prebiotika, anti-cancer egenskaber og til anvendelse som afgiftning af tungmetaller (Hotchkiss, Rastall, Gibson, Eliaz, Liu, & Fishman, 2009). Solsikke pektin blev derfor ekstraheret og fraktioneret efter størrelse SPF<50, SPF50 og SPF>100 ved hjælp af 50 og 100 kDa membraner. Densiteten af *Bifidobacterium* spp. var betydelig højere ($p<0.05$) efter fermentering af SPF>100. De tre pektiner fra solsikke gav den sameffekt på de tre andre mave-tarm bakterier, *Lactobacillus* spp., *Bacteroidetes* and *Firmicutes*.

List of publications

1. Application of enzymes for efficient extraction, modification and development of functional properties of lime pectin.
Malgorzata Dominiak, Karen M. Søndergaard, Jesper Wichmann, Silvia Vidal-Melgosa, William G.T. Willats, Anne S. Meyer, Jørn D. Mikkelsen (2014). Food Hydrocolloids, 40, 273-282.
2. FTIR, carbohydrate microarray and multivariate analysis for assessing pectin extracts.
Andreas Baum, Malgorzata Dominiak, Silvia Vidal-Melgosa, William G.T. Willats, Karen Marie Søndergaard, Per Waaben Hansen, Anne S. Meyer, Jørn Dalgaard Mikkelsen
Submitted
3. Evaluation of the prebiotic potential of sunflower pectin fractions.
Malgorzata Dominiak, Louise K. Vignæs, Tine R. Licht, Karen M. Søndergaard, Jørn D. Mikkelsen
Presented as manuscript

Abbreviations

ANOVA	One-way analysis of variance
Ca ²⁺	Calcium ion
Ct	Cycle number
CBM	Carbohydrate binding modules
DA	Degree of amidation
DAC	Degree of acetyl esterification (acetylation)
DE	Degree of esterification
DM	Degree of methyl esterification (methylation)
DP	Degree of polymerization
EDTA	Ethylene diaminetetraacetate
Endo-PG	Endo-acting PG
Exo-PG	Exo-acting PG
FOS	Fructo-oligosaccharides
f-PME	Fungal-PME
FTIR	Fourier transform infrared spectroscopy
Gal	Galactose
GalA	α -D-galactopyranosyluronic acid
GPC	Gel permeation chromatography
HE-pectin	High ester pectin
HG	Homogalacturonan
HM-pectin	High methyl ester pectin
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
LM pectin	Low methyl ester pectin
MALLS	Multi-angle laser light scattering
MBM	Minimal basal medium
MSNF	Milk solid non-fat
Mw	Molecular weight
NEG	Negative control
PBS	Phosphate buffered saline
PAT	Process analytical technologies

PCA	Principal component analysis
PCs	Principal components
PG	Polygalacturonase
PGA	Polygalacturonic acid
PL	Pectin lyase
PLS	Partial least squares
PME	Pectin methylesterase
p-PME	Plant-PME
qPCR	Quantitative polymerase chain reaction
Q^2	Predictivity
RGI	Rhamnogalacturonan I
RGII	Rhamnogalacturonan II
Rha	Rhamnose
RMSEP	Root mean square error of prediction
RPM	Rounds per minute
R^2	Correlation coefficient
RI	Refractive index
SEC	Size exclusion chromatography
SNV	Standard normal variate
SPF<50	Sunflower pectin fraction of $M_w < 50$ kDa
SPF50-100	Sunflower pectin fraction of M_w between 50 and 100 kDa
SPF>100	Sunflower pectin fraction of $M_w > 100$ kDa

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1. Introduction

The refinery of biomass to recover labile, value-added components is an important future requirement for the utilization of side-streams from the agricultural and dairy industries. Presently, most of the processes using biomass are exclusively focused on the total hydrolysis of polymers to form fermentable sugars for bio-ethanol production. Commercial enzymes are able to break down most plant cell wall materials, but there is very little incentive to generate selective processes with several product lines, where value-added, labile products could be recovered before residual biomass is used for bioethanol production. It is, however, not a simple task to integrate an up-stream process, where labile polymers like hydrocolloids could be harvested before degradation of the residual biomass to mono-saccharides.

The current pectin production method employs high temperature (70-90°C) in combination with acidic hydrolysis using nitric, hydrochloric or sulfuric acid. The pH is between 1.5 and 2.5, and the reaction is continued for several hours (Rolin, Nielsen, & Glahn, 1998). The main disadvantage of acid hydrolysis technology, and one which raises environmental concerns, is the generation of large volumes of acidic effluent that require further treatment before release. Moreover, harsh acidic treatment causes depolymerization and deesterification of the pectin chains. Enzymatic extraction of pectin seems more advantageous in terms of energy consumption and waste management. The enzymatic process is usually carried out at a pH between 3-5 and temperatures around 50°C, which is more beneficial in terms of economy and environmental impact. There have been a few attempts to extract pectin with enzymes described in the literature. They involved the use of endo-arabinases and endo-galactanases (Thibault et al., 1998), cellulases together with proteases (Zykwinska et al., 2008), cellulase together with pectin esterase (Ptichkina, Markina, & Rummyantseva, 2007) or only cellulase (Yuliarti et al., 2011). The problems associated with enzymatic extraction were, however, low pectin yields, low molecular weights of the product and low galacturonan content.

Pectin production is complex and therefore its optimization is a long process, because the evaluation of the final product quality is accomplished at the end of the procedure, employing time-consuming off-line laboratory tests. Therefore, it would be of interest to pectin producers to shift the quality control upstream in the process. Nowadays, there is a tendency to apply Process Analytical Technologies (PAT) for on-line process monitoring and control. PAT involves the use of suitable sensor technologies, statistical tools, e.g. chemometrics, and feedback process control to understand the impacts of different variables on

product quality. Such an approach is desirable for the production of pectin, which is currently based on post-process product testing.

Although the major applications of pectin still include gelling, thickening and stabilization, other novel applications employ its prebiotic potential, anti-cancer properties and heavy metal detoxification ability (Hotchkiss et al., 2009). The field of prebiotics is expanding rapidly and new prebiotic candidates are constantly being investigated. Prebiotic ingredients are developed with the help of enzymes, modern molecular methods and advanced process knowledge. By exploring new prebiotic sources, molecules with new functionalities are found. One such candidate could be sunflower pectin, present in head and stalk residues of sunflower plants after removal of oil-rich seeds. The head and stalk residue wastes contain 6.5% proteins, 3.1% lipids, and 71.9% carbohydrates, such as cellulose, hemicelluloses and pectin (Miyamoto & Chang, 1992) that could be further extracted. Sunflower pectin content in the heads varies between 15 and 24% and after extraction the pectin contains between 29 and 40% methyl groups (Lin, Sosulski, & Humbert, 1978; Miyamoto & Chang, 1992), which makes it an interesting alternative to traditional citrus or apple pectins and a potential prebiotic candidate.

1.1 Hypotheses and objectives

Hypotheses:

1. Enzymes can catalyze the release of pectin with superior yields, viscosities and functional properties to those of acid extracted pectin.
2. FTIR and carbohydrate microarrays are useful tools for the prediction of pectin yields during pectin production processes and for characterization of crude pectin extracts.
3. Prebiotic compounds can be derived from sunflower pulp and their sizes are of importance for their prebiotic responses.

Specific objectives:

1. Optimize enzymatic catalysis for the release of lime pectin. Test the applicability of the product pectin as a food ingredient and compare its performance with pectins obtained in the traditional way.
2. Apply FTIR and carbohydrate microarrays combined with chemometrics for characterization of crude enzymatically extracted pectin versus acidically extracted pectin.
3. Release the pectic substances from sunflower pulp in high yields and test their biofunctionality by using *in vitro* fecal fermentations.

1.2 Plant cell wall architecture

The cell walls of dicotyledonous plants consist of primary and secondary walls. Primary walls, formed in developing cells, are predominantly composed of cellulose, hemicellulose, and pectin. They are non-lignified and their thickness in mature cells depends on the cell type. Secondary walls are deposited on the primary walls after the cells are fully expanded and are often rigidified by lignin. The interfacial layer between adjacent cells, the middle lamella, is also typically lignified (Harris & Stone, 2008).

1.2.1 Structural domains of plant cell walls

Primary cell walls consist of three interacting structural domains: cellulose-xyloglucan frameworks (about 50% of the wall mass), pectic polysaccharides (about 30% of the total mass) and structural proteins (Carpita & Gibeaut, 1993). A schematic representation of a cell wall is presented in Figure 1.

Cellulose-xyloglucan framework contains a network of cellulose microfibrils, which are linear chains of 1,4- β -linked D-glucose in the form of long crystals (Carpita & Gibeaut, 1993). The 1,4- β -linked D-glucose chains are typically several thousand units long (Marx-Figini, 1982; Timpa, 1991) and begin at different places within a microfibril. Cellulose microfibrils are interlocked with xyloglucan polysaccharides, which consist of linear chains of 1,4- β -D-glucan. The 1,4- β -D-glucan chains are frequently substituted with xylosyl units at the *O*-6 position and less frequently substituted with β -D-galactose and α -L-arabinose at the *O*-2 of xylosyl units (Carpita & Gibeaut, 1993). Other non-cellulosic polysaccharides, such as gluco- and galactoglucomannans, galactomannans, 1,3- β -D-glucans, and glucuronoarabinoxylans, are found in much lower amounts in the cell wall (Maltby et al., 1979).

Pectic networks contain three major forms of pectin: homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). The major component of all the forms of pectin is a linear chain of D-galacturonic acid units, in which varying proportions of the acid groups are present as methyl esters (more detailed information in Chapter 1.3).

Proteins make up to 10% of the dry weight of the wall and include enzymes, signaling molecules and structural proteins, which are quantitatively the most significant. Extensin is probably the best-described structural protein in plants and consists of a repeating serine-hydroxyproline, and tyrosine-lysine-tyrosine sequences (Harris & Stone, 2008).

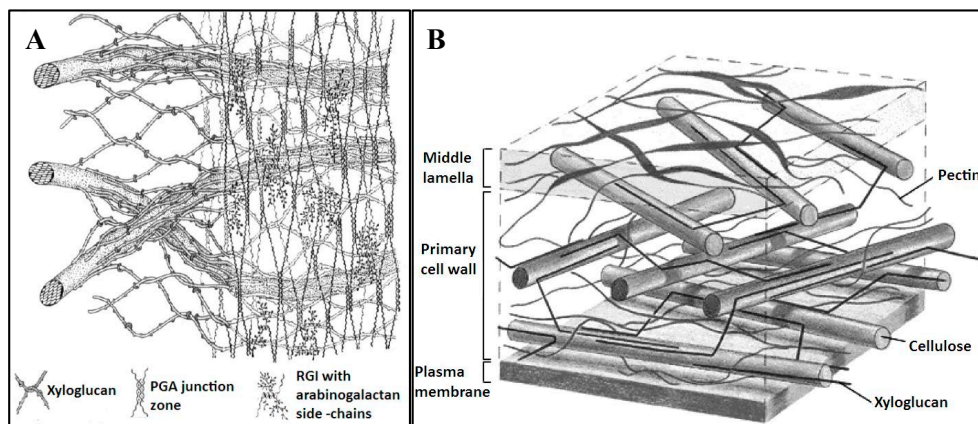


Figure 1. Schematic representation of the spatial arrangements of the polymers in the cell walls of flowering plants adapted from (A) Carpita & Gibeaut, (1993); (B) McCann & Roberts (1991).

1.2.2 Enzymatic degradation of the cell walls

Efficient conversion and utilization of plant cell wall carbohydrates is of major importance in biotechnology. The process of plant cell wall degradation is complicated due to the complexity of cell walls and the variety of bond types which need to be broken. Therefore, complex mixtures of degradative enzymes are needed to break down the polysaccharide structures. The extent of recalcitrance of cell walls to degradation by enzymes depends on the ability of enzymes to access the substrates in the cell walls (Harris & Stone, 2008). Accessibility is related to the surface area of the cell wall that is exposed to the hydrolytic agent and the complexity of the cell wall. The presence of lignin has a particularly strong effect on the digestibility of plant cell walls. In most cases, non-lignified walls are highly degradable, while lignified walls are less susceptible to degradation. Environmental factors affecting plant growth, such as lack of nutrients, temperature deviations or water shortage, also impact cell wall digestibility (Buxton & Casler, 1993).

Polysaccharides present in plant cell walls are degraded by the plant's endogenous enzymes during fruit ripening, seed germination and cell wall extension. Enzymes associated with the biochemical processes of cell wall degradation in the plant include endo- and exo-polygalacturonases (endo- and exo-PG), cellulase, glycosidases, and pectin methyl esterases (PMEs) (Ward & Moo-Young, 1989). During degradation, pectic substances undergo changes, such as demethylation, deacetylation and shortening of the polymer chain. Many extracellular enzymes that degrade plant cell walls are produced by industrial

microorganisms, particularly fungi, which grow on complex plant materials, namely *Aspergillus*, *Penicillium*, *Rhizopus* and *Trichoderma* sp. (Ward & Moo-Young, 1989). Enzyme production processes, which utilize industrial microorganisms are optimized to produce a particular enzyme activity and products are standardized with respect to one or a small number of activities. As a result, side activities are present in commercial enzyme preparations and are often important for effective cell wall degradation. The modes of action of enzymes performing side activities are, however, often not known and not optimized.

Although there have been significant advances in the structural analysis of plant cell wall-degrading enzymes, still only a small percentage of the enzymes in CAZy database are characterized biochemically and structurally (Gilbert, 2010). The biochemical properties of plant cell wall-degrading enzymes are difficult to evaluate due to the chemical complexity of the cell wall and the requirement for a hierarchical and synergistic hydrolytic processes.

1.3 Structural features of pectin

Pectic polymers are the most complex and diverse group of cell wall polysaccharides and have a range of functions in plant tissue including: size and shape determination, integrity control, water holding capacity, ion transport, ‘cementing’ and cell adhesion (Basic, Harris, & Stone, 1988; Rees, & Wright, 1969; Voragen, Beldman, & Schols, 2001; Willats, McCartney, Mackie, & Knox, 2001). The term ‘pectic substances’ is derived from the Greek ‘pektikos’ meaning to congeal or solidify (Willats et al., 2006). Pectic substances include pectic acids esterified with methanol, the de-esterified pectin form and its salts, as well as neutral polysaccharides, such as arabinans, galactans and arabinogalactans. The term ‘protopectin’ denotes the native form of pectin in the cell wall, before its extraction in a ‘degradative’ way which affects the structure (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995).

1.3.1 Structural domains of pectin

1.3.1.1 Galacturonans

Homogalacturonan (HG) is the dominant type of pectin in lime peel and consists of a linear chain of α -1,4-linked galactopyranosyluronic acid (GalA) residues, being partly methyl-esterified at *O*-6 position and sometimes acetyl-esterified at *O*-2 or *O*-3 (Voragen et al., 2001). The presence of HG-domain is crucial for gel formation (Voragen et al., 1995). Partly esterified or non-esterified HG domains form the

so called ‘smooth regions’ of the pectin chain, whereas the substituted domains form the ‘hairy regions’ (Figure 2).

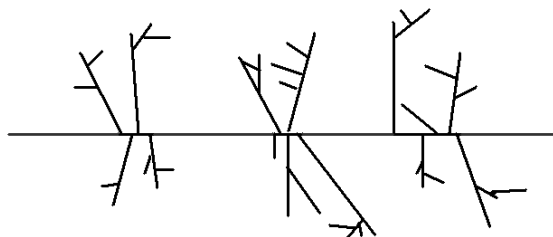


Figure 2. Schematic representation of pectin molecule with smooth (grey) and hairy (black) regions.

Rhamnogalacturonan II (RGII) is composed of a linear backbone chain of GalA, substituted with L-Rha, D-Gal (galactose) and many unusual sugars, such as apiose, 3-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, 3-*C*-carboxy-5-deoxy-L-xylose, 3-deoxy-D-manno-octulosonic acid and 3-deoxy-D-lyxo-heptulosaric acid (Mohnen, 2008; Voragen et al., 2001). The side chains of RGII consist of 12 different types of sugars with over 20 different linkages. The structure of RGII, the most structurally complex pectin domain, is largely conserved across many plant species. Cross-linking between RGII chains of two adjacent pectin molecules increases the integrity of the pectin network (Mohnen, 2008).

Xylogalacturonan is a homogalacturonan substituted at *O*-3 with a β -linked D-xylose, which is in turn occasionally substituted at *O*-4 with an additional β -linked xylose (Zandleven, Beldman, Bosveld, Schols, & Voragen, 2006). The proportion of xylopyranosyl residues to galactosyluronic acid residues ranges from 40 to 90% (Mohnen, 2008). The galacturonan backbone may also be methyl esterified independently of the xylose substitutions. Xylogalacturonans in plant tissues are associated with functions including storage and reproduction (Schols, & Voragen, 1996).

Apiogalacturonans are homogalacturonans with mono- and di- D-apiose substitutions. Apiogalacturonans have previously been isolated from the cell walls of the aquatic duckweed and sea grass (Ovodov, 1975).

1.3.1.2 Rhamnogalacturonan I

RGI, composed of a repeating disaccharide units: 1,2-L-Rha-1,4-D-GalA (where Rha denotes rhamnose), is abundantly substituted by arabinan, galactan and arabingalactan side chains, predominantly at the *O*-4 position of rhamnose (Schols, & Voragen, 1994; Yapo, Lerouge, Thibault, & Ralet, 2007), as presented in Figure 3A. RGI backbone may contain up to 300 rhamnosyl and 300 galactosyluronic acid residues (Voragen et al., 2001).

Type I is a linear chain of 1,4-linked β -D-galactose, containing up to 25% α -L-arabinose residues 1,5-linked in short side chains, connected predominantly to *O*-4 of the rhamnosyl residues (Voragen et al., 2001). Single galactose substitutions at *O*-6 have also been found. Pectin with type I arabinogalactans have been isolated from citrus, potato, soybean, lupin, apple, onion, kiwi, tomato and cabbage (Voragen et al., 1995). Arabinogalactan type II contains ramified chains of β -D-galactose, joined predominantly by 1,3-linkages in the interior chains, and mainly by 1,6-linkages in the exterior chains. Type II arabinogalactan is a component of arabinogalactan proteins (Basic et al., 1988).

Although it is generally accepted that HG and RGI domains form the ‘backbone’ of pectin, as shown in Figure 4A, an alternative structure has been proposed by Vincken et al. (2003). In this model HG is a long side chain of RGI (Figure 4B).

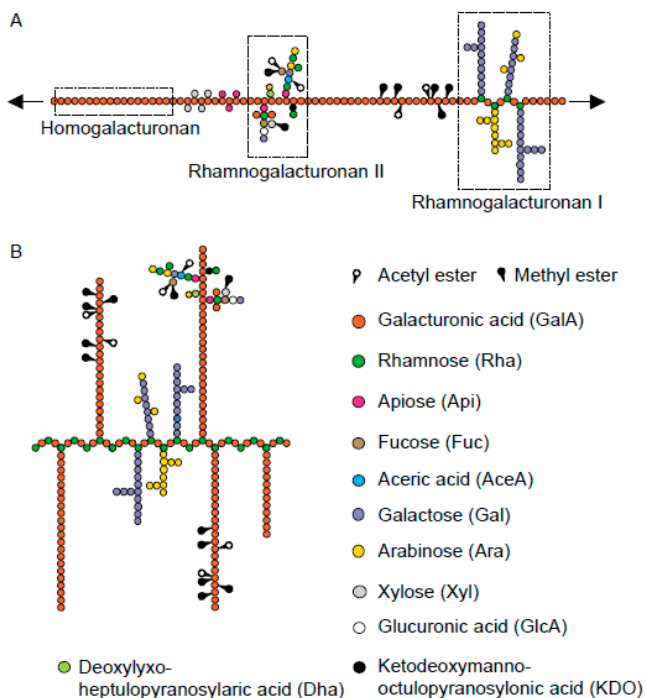


Figure 4. Schematic representations of the conventional (A) and alternative (B) structures of pectin. Figure adapted from Willats et al. (2006).

1.3.2 High ester, low ester and amidated pectins

The presence of non-sugar substituents, methyl and acetyl groups, is a very important feature of pectic polymers, affecting their functionalities. Pectin producers traditionally divide pectin polymers into high- and low-methoxyl pectins (HM and LM pectins). The boundary level between the two types is 50%, meaning that if more than 50% of a pectins carboxyl groups are methylated, it is termed HM pectin, and if this proportion is below 50%, it is LM pectin.

The degree of acetylation (DAc) of pectin is defined as the percentage of galacturonic acid residues in the pectin that are esterified with acetyl groups. Acetyl groups are present in very low amounts in HG from apple and citrus, but in much higher amounts in HG from sugarbeet and potato. Acetylated pectin does not have the ability to form gels with Ca^{2+} ions, but possess emulsion-stabilizing properties (Voragen et al., 2001).

LM pectins are often chemically amidated by manufacturers to change the polymer functionality. The degree of amidation (DA) is then defined as a percentage of amidated galacturonic acid residues within pectin (Voragen et al. 1995).

1.3.3 Molecular weight (Mw) and aggregation

The molecular weight (Mw) of pectins depends on the raw material and the conditions applied during production. Pectin samples are heterogeneous and the molecules have a tendency to aggregate, which makes determination of their molecular weights difficult. Viscosimetry is often employed for determination of molecular weight of pectin samples. The results usually fall in the range 50 to 150 kDa. Other techniques, such as light scattering, often result in apparent molecular weights of around 1000 kDa, as they are more sensitive to the presence of aggregates (Sawayama, Kawabata, Nakahara, & Kamata, 1988; Sorochan, Dzizenko, Bodin, & Ovdov, 1971; Fishman, Chau, Kolpak, & Brady, 2001). Additionally, pectin samples often contain a small fraction of high Mw pectins, which impact light scattering measurements but have almost no effect on viscosity (Berth, 1988).

The phenomenon of pectin aggregation in solution was first recognized more than 40 years ago (Sorochan, Dzizenko, Bodin, & Ovdov, 1971). Pectin molecules display a tendency to aggregate when dispersed in water. Whether pectin aggregates depends on ionic strength and pH of the solution and the presence of solvent additives (Sorochan et al., 1971; Sawayama, Kawabata, Nakahara, & Kamata, 1988).

Aggregation is promoted by complex forces resulting from a combination of gel formation, precipitation and solubilization mechanisms (Yoo, Fishman, Savary, & Hotchkiss, 2003), and the presence of aggregates is responsible for the increase of apparent size of pectin molecules. Pectin aggregates have been investigated by many authors using electron microscopy (Hanke & Northcote, 1975; Fishman, Cooke, Hotchkiss, & Damert, 1993), membrane osmometry, end-group analysis (Fishman, Pepper, & Pfeffer, 1986), and high performance size exclusion chromatography (HPSEC) (Fishman, Chau, Hoagland, & Ayad, 2000).

Using electron microscopy, Fishman et al. (1992) observed aggregated spherical network structures in peach pectin water solutions, which could be dissociated into linear structures by the addition of the hydrogen bond breakers, NaCl and glycerol (Fishman et al., 1993). Cárdenas, Higuera-Ciapara and Goycoolea (1997) reported an increased Mw of cactus mucilage (referred to as a pectin polysaccharide) due to the formation of large macromolecular aggregates that were unable to dissociate during permeation through the gel beads during SEC-HPLC. Fishman et al. (2001) reported that a series of commercial pectins had higher Mws when dissolved in NaNO₃ than in LiAc/HAc, when Mws were measured by HPSEC with on-line light scattering. The increased Mws in NaNO₃ suggest that pectins were more aggregated in NaNO₃ than in LiAc/HAc.

Yoo, Fishman, Hotchkiss, & Lee (2006) investigated the intrinsic viscosities of high and low methoxy-pectins at different salt concentrations and observed the presence of stronger aggregates in the case of LM pectin. Stronger aggregation by LM pectins was explained by the fact that the primary driving force for aggregation is hydrogen bonding and the larger number of carboxyl groups in LM-pectin than in HM-pectin produced more hydrogen bonds and therefore stronger aggregates. The authors also distinguished two types of pectin aggregation: i) lateral chain aggregation of LM-pectin at concentrations lower than 0.0004 g/ml, which was interrupted by high salt concentration, and ii) end to end chain aggregation at a concentration above 0.0004 g/ml leading to gelation. Other authors suggested that the increase in Mw of LM pectin occurred due to transacylation activity of PME (Jiang, Lai, Chang, & Chang, 2001).

Oakenfull & Scott (1984) suggested that the primary forces responsible for HM pectin aggregation are hydrogen and hydrophobic bonding. Paoletti, Cesaro, Delben, & Ciana (1986) reported the formation of chain-chain aggregates, especially at low pH. Sawayama et al. (1988) observed a 25 fold increase in the Mw of polygalacturonic acid accompanying a decrease in pH from 4.4 to 2.35 (Figure 5).

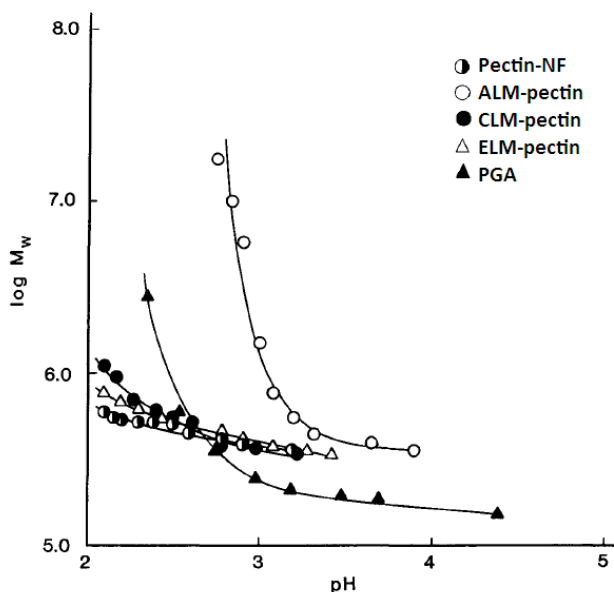


Figure 5. Effect of pH on the Mw of pectins with different functional groups. Pectin-NF is a HM pectin and the other three are low ester, obtained from pectin-NF by ammonia (ALM), acid (CLM) and pectin methyl esterase from *Aspergillus japonicus* (ELM) deesterification. PGA denotes polygalacturonic acid. Figure adapted from Sawayama et al. (1988).

1.3.4 Stability

Conditions that render pectin stable or unstable are important when considering pectin extraction processes. The conditions used during extraction must allow pectin molecules to remain stable in order to prevent damage during the extraction process. Pectin in solution is most stable at pH between 3 and 4. At temperatures above 60°C and pH below 5, glycosidic bonds at the non-reducing end of a methylated galacturonic acid residue undergo β -elimination and the pectin chains become depolymerized. Therefore, HM pectins are quite vulnerable to high pH. At pH below 3, even at low temperatures, ester linkages are broken and neutral sugar side chains are hydrolyzed. Low pH and elevated temperatures cause hydrolysis of the galacturonan backbone (Albersheim, Neukom, & Deuel, 1960; May, 1990).

1.3.5 Pectin-degrading enzymes

Pectin is susceptible to enzymatic degradation by endogenous and exogenous enzymes (Figure 6). Endogenous enzymes can cause changes in the plant during ripening and storage. Exogenous microbial and fungal enzymes disintegrate pectin and play a role in plant pathology (Voragen et al., 1995). Once the pectin containing fruit is processed, the pectin is under attack of those enzymes. Therefore, it is essential to either release the pectin fast from fresh materials, or to stabilize the pulp or pomace by drying.

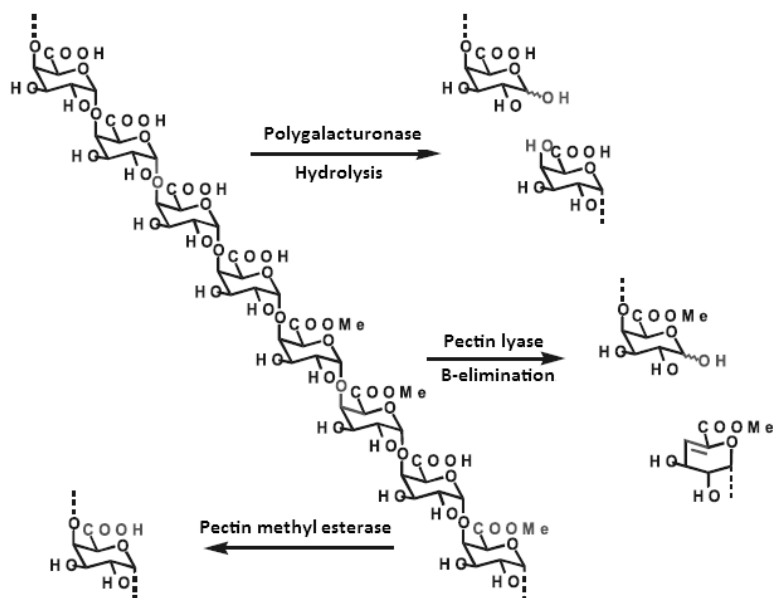


Figure 6. Enzymatic degradation of pectin by endo-polygalacturonase, pectin lyase and pectin methyl esterase. Figure adapted from Lundt, Madsen, Christensen and Mikkelsen (2002).

The main enzymes that catalyze the breakdown of HG pectin domains are listed in Table 1.

Table 1. Pectolytic enzymes active against pectin HG domain.

Enzyme	EC number	Substrate	Action
Polygalacturonase	EC 3.2.1.15	LM pectins, pectic	Hydrolysis of glycosidic bonds adjacent to free carboxyl group
	EC 3.2.1.67	acid	
Pectate lyase	EC 4.2.2.2	LM pectins, pectic	β -eliminative breakdown of glycosidic bonds adjacent to free carboxyl group
	EC 4.2.2.9	acid	
Pectin lyase	EC 4.2.2.10	HM pectins	β -eliminative breakdown of glycosidic bonds adjacent to methoxylated carboxyl group
Pectin methyl esterase	EC 3.1.1.11	Methylated HG	Hydrolysis of ester bonds
Pectin acylesterase	EC 3.1.1.-	Acetylated HG	Hydrolysis of ester bonds

Polygalacturonases can be divided into endo- and exo- acting. Endo-polygalacturonases act inside the HG chain, whereas exo-polygalacturonases act from non-reducing end of the HG chain and release monomers and dimers of galacturonic acid (Voragen, 1995). Enzymes active in the degradation of RGI domains of pectins include rhamnogalacturonan hydrolase (EC 3.2.1.-), rhamnogalacturonanlyase (EC 4.2.2.-) and rhamnogalacturonan acetyl esterase (EC 3.1.1.-). Other enzymes, including galactanases and arabinases, act towards pectin side chains.

1.3.6 Enzymatic fingerprinting of pectin

The availability of purified, highly specific enzyme preparations has significantly contributed to the structural characterization of pectins by so called enzymatic fingerprinting. Enzymes can be used to disintegrate the long and highly complex pectin chains at certain positions, and the generated products can be structurally characterized by e.g. chromatography and/or mass spectrometry (MS) (Limberg et al., 2000a; Körner et al., 1999).

Using enzymatic fingerprinting Limberg et al. (2000a) studied the de-esterification mechanisms of fungal and plant pectin methyl esterases (f- and p-PME) together with base de-esterification. Pectins with different distributions of methyl groups were produced and enzymatically digested. The products were analyzed using matrix assisted laser desorption ionization mass spectrometry (MALDIMS) and high-performance anion-exchange chromatography with pulsed amperometric or UV detection (HPAEC–PAD:UV). P-PME and f-PME were shown to produce significantly different deesterification patterns, as revealed by the analysis of the oligomers after digestion. The action of p-PME resulted in the consecutive removal of a number of neighboring methyl ester groups, whereas f-PME removed methyl groups in a random fashion. MALDIMS identified the preferred substrates for pectin lyase (fully methyl esterified HG) and polygalacturonase (HG without any methyl esterification).

In a subsequent publication, Limberg et al. (2000b) developed a method for pectin characterization based on endo-PG and exo-PG digestion and subsequent quantification of the amount of galacturonic acid residues in block sequences. The authors showed that endo-PG and exo-PG only acted between two free galacturonic acid units (Figure 7). Measurement of the increase in galacturonic acid residues in block sequences during de-esterification provided an insight into different methods used for de-esterification.

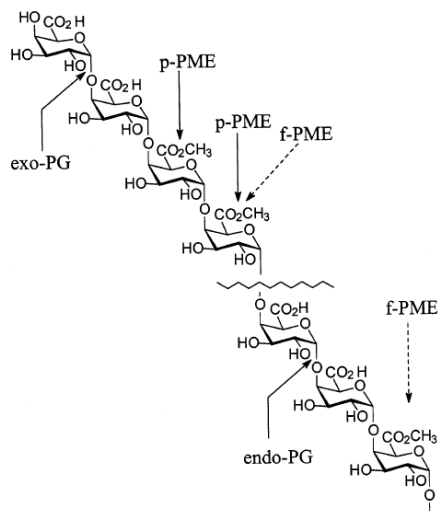


Figure 7. Specificities of pectinolytic enzymes on a partly methylesterified HG backbone. Figure adapted from Limberg et al. (2000b).

Körner, Limberg, Christensen, Mikkelsen, and Roepstorff (1999) digested partially methyl-esterified pectin with PL, endo-PG, and exo-PG and studied the esterification patterns of the obtained oligomers by collision-induced dissociation in a nanoelectrospray ionization ion trap mass spectrometer. Methyl esterification patterns provided insight into substrate specificities of the enzymes used for digestion.

Remoroza et al. (2012) degraded sugarbeet pectin using endo-PG, PL, f-PME and RG-I degrading enzymes. Hydrophilic interaction liquid chromatography (HILIC) with online electrospray ionization ion trap mass spectrometry (ESI-IT-MS_n) and evaporative light scattering detection (ELSD) were used for separation, identification and quantification of the released oligomers. MS enabled elucidation of the structures of the acetylated oligomers eluted from the HILIC column. The presence of methyl and acetyl groups reduced the interaction of the oligomers with the HILIC column, as compared with the unsubstituted oligomers.

Since pectin is highly complex and heterogeneous, methods like enzymatic fingerprinting play an important role in the identification of structural motifs of pectin and elucidation of the overall pectin structure. Furthermore, such techniques can be applied for prediction of the functional properties of pectins.

1.4 Pectin as a functional food ingredient

Pectin is used in the food industry as an ingredient due to its functional properties including enabling gelling, providing viscosity, stabilizing proteins and acting as a fat mimetic (Voragen et al., 1995; Voragen et al., 2001; Willats, Knox, & Mikkelsen, 2006). The major applications of pectin in the food industry include gelling and thickening of jams, jellies, bakery fillings, confectionery and fruit preparations for dairies, as well as stabilization of milk and fruit beverages (Rolin, Nielsen, & Glahn, 1998). Other novel pectin applications employ its prebiotic potential, anti-cancer properties and heavy metal detoxification ability (Hotchkiss et al., 2009). All of the above-mentioned features result in a constant increase in worldwide pectin consumption (Ptichkina, 2007).

1.4.1 Gelling

Traditionally, gelation is considered in the contexts of HM and LM pectins, but in fact there are many gelation mechanisms that act together to form a continuous three-dimensional network of cross-linked pectin molecules. Apart from the degree of methylation, gelation is also dependent on the distribution of the ester groups along the chain and the average pectin Mw. Other factors such as solution pH, ionic strength, sugar content and temperature also play a role in gel formation (Thakur, Singh, & Handa, 1997).

HM pectins gel at $\text{pH} < 3.5$ and in the presence of at least 55% sugar. Addition of sugar reduces water activity in the system and promotes hydrophobic interactions between methyl ester groups (Oakenfull & Scott, 1984). Low pH decreases the repulsion between pectin molecules and supports the formation of hydrogen bonds between pectin chains. The increase in hydrophobic interactions and hydrogen bonds, presented in Figure 8, is responsible for gel formation.

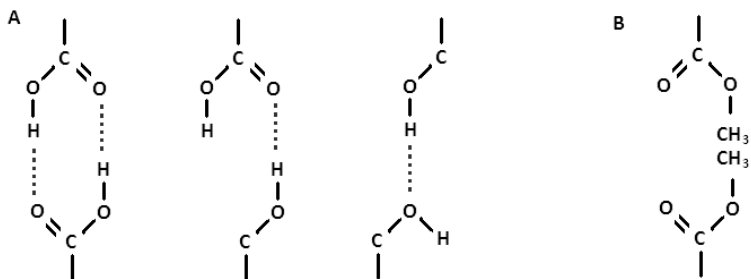


Figure 8. Hydrogen bonds (A) and hydrophobic interactions (B) responsible for gelling of HM pectin.

In LM pectins, gelation is initiated by the formation of calcium bridges between free carboxyl groups (in addition to other mechanisms). The junction zones form the so called ‘egg boxes’, as shown in Figure 9 (Grant, Morris, Rees, Smith, & Thom, 1973). The pH of the gel could be higher than in case of HM pectins, as only the dissociated carboxyl groups form calcium bridges.

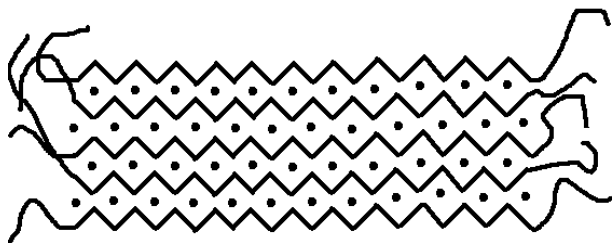


Figure 9. Schematic representation of an ‘egg box’ gelling of LM pectin. Black dots represent Ca^{2+} ions.

1.4.2 Stabilization of proteins using pectins

In acidified milk drinks, casein particles have a tendency to aggregate and sediment, a problem which is increased by heat treatments intended to increase milk shelf life. As a result, the casein separates and the product becomes sandy. When HM pectin is added to the system, it adsorbs onto the surface of the casein molecules due to the electrostatic interactions between the negatively-charged pectin polymer and the positively charged casein particles (Figure 10). This interaction is only present at pH between 3.6 and 4.5. At pH below 3.5, the pectin does not carry sufficient negative charge to allow it to adsorb to the surface of the casein and at pH above 4.6 (the isoelectric point of casein), the casein particles become negatively charged and the complex becomes too weak (Rolin et al., 1998).

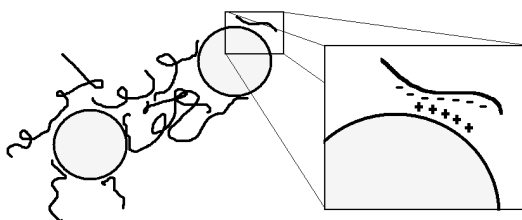


Figure 10. Stabilization of protein molecules by a pectic network.

The adsorption of pectin on the surface of casein micelles requires the presence of blocks of unesterified carboxyl groups within the pectin polymer. The uncharged parts of pectin chain form loops that cause steric stabilization of the complex (Tromp, de Kruif, van Eijk, & Rolin, 2004). Pectin addition causes an increase in the viscosity of the milk drink and increases its stability. For optimum stabilization, the pectin

needs to have a high Mw and high degree of esterification. The distribution of methyl esters is also important. Too large blocks of unesterified galacturonic acid tend to interact with ions rather than proteins, leading to increased viscosity and gelation. Therefore, LM pectins are not useful for this application. Moreover, it has been found that up to 90% of the pectin added to the milk drink is not interacting directly with casein, but is still necessary to produce a stable drink during homogenization. This is the so-called ‘serum pectin’ (Tromp et al., 2004). The suitability of pectin for stabilization of acidified milk drinks is assessed by the measurement of sediment as a function of pectin dose (Figure 11).

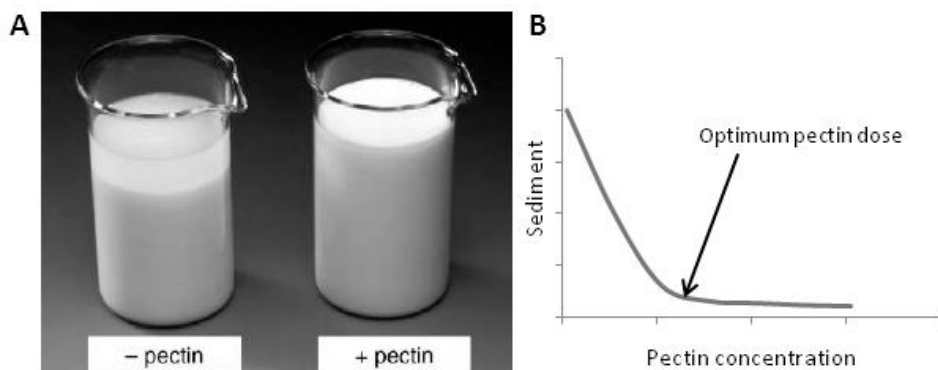


Figure 11. Pectin and milk stabilization. (A) Visible sedimentation of casein particles in an acidified milk drink with and without pectin (figure adapted from Lundt et al., 2002). (B) The assessment of the suitability of pectin for stabilization of proteins in acidified milk drinks. Milk drinks with different pectin concentrations are prepared and they are assessed, after a defined storage period, for the presence of sediment. The lowest pectin concentration, at which no sediment is observed, is defined as the optimum pectin dose.

1.5 Bioactivity of pectin

Pectins have been reported to exert a positive impact on the human gastrointestinal tract, due to their water-holding capacities and gelling abilities (Roberfroid, 1993). The positive effects of pectin on the human gastrointestinal tract include slowing down gut transit and delaying gastrointestinal emptying (Flourineet al., 1985; Spiller et al., 1980), as well as decreasing glucose absorption (Jenkins et al., 1977). Pectin passes through the upper gastrointestinal tract to the large intestine in its intact form and is extensively fermented in the colon (Olano-Martin, Mountzouris, Gibson, & Rastall, 2001). Low ester pectins have been shown to be degraded faster than high ester pectins (Dongowski & Lorenz, 1998).

1.5.1 Prebiotic potential

A prebiotic is a non-digestible food ingredient that is able to selectively enhance the growth and activity of the gut microflora that benefits the host (Scantlebury Manning, & Gibson, 2004; Thammarutwasik et al., 2009; Wichienchot et al., 2011). These beneficial effects include immunostimulation, improved mineral absorption, cholesterol lowering, vitamin synthesis and colon cancer prevention. Typical

prebiotics are short-chain carbohydrates, such as fructo-oligosaccharides (FOS, produced from fructose *via* biosynthesis or from inulin *via* hydrolysis) or polysaccharides, such as inulin or levan (Gibson, 1995). Industrial prebiotics are either naturally occurring oligosaccharides or are produced by enzymatic degradation of raw materials.

Evaluation of prebiotic potential is based on the selective stimulation of growth of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* strains, and/or a decline in the growth of pathogenic bacteria like *Clostridium* (Olano-Martin, Gibson, & Rastall, 2002; Hotchkiss, Olano-Martin, Grace, Gibson, & Rastall, 2003; Holck et al. 2011a; Thomassen, Vign  s, Licht, Mikkelsen, & Meyer, 2011). Previous studies have focused on the modification of the ratio between *Bacteroidetes* and *Firmicutes*, two dominant human intestinal phyla, which may be related to the development of obesity (Licht et al. 2010; Holck et al. 2011b). Studies of prebiotic potential have been conducted with both pure cultures and with human mixed fecal cultures.

Pectins have previously been investigated for their prebiotic potentials. Production of pectin-derived oligosaccharides from plant biomass typically involves pretreatment of cell-wall material, extraction of pectin polysaccharides, generation of oligosaccharides and their purification by chromatography (Holck et al., 2011a). Pectins were found to promote the growth and activity of bifidobacteria, inhibited pathogen growth and caused bowel cancer cells apoptosis (Hotchkiss, Olano-Martin, Grace, Gibson, & Rastall, 2003). Olano-Martin, Gibson and Rastall (2002) found that oligosaccharides derived from LM and HM pectins had more prebiotic activity than the pectins that they were derived from. Moreover, LM oligosaccharides were more prebiotic than HM oligosaccharides. Furthermore, pectin size also had an effect on prebiotic properties, indicating that the prebiotic potential of pectin could be improved by hydrolysis.

Holck et al. (2011a) investigated the prebiotic potential of oligosaccharides derived from sugar beet pectin. A series of highly purified homogalacturonides and rhamnogalacturonides were prepared by step-wise enzymatic degradation. The ratio of *Bacteroidetes* to *Firmicutes*, which is believed to play a role in the development of obesity, was different during *in vitro* fermentations using homogalacturonan oligos with DP4 and DP5, indicating that a small difference in the structure of the prebiotic affects the biological activity of the phyla. In another study, Holck et al. (2011b) selectively stimulated bifidobacteria in *in vitro* fermentations using feruloylated and the non-feruloylated long-chain arabino-oligosaccharides.

Thomassen, Vignæs, Licht, Mikkelsen, & Meyer (2011) released fibers from potato pulp and examined their prebiotic effects. Two broad fractions were obtained: 10–100 kDa and >100 kDa, both were found to be more bifidogenic than the well-established prebiotic, FOS. Moreover, the densities of *Bifidobacterium* spp. and *Lactobacillus* spp. increased 2–3 times, compared to FOS, in the case of fibers with molecular masses above 100 kDa.

1.5.2 Anti-cancer properties, heavy metal detoxification and bacterial adhesion.

According to several studies, pectin-derived oligosaccharides have the capacity to prevent a number of severe chronic diseases. Low Mw pectin has been shown to induce apoptosis in colon and prostate cancer cells (Olano-Martin et al., 2003a; Jackson et al., 2007), as well as increased the life quality and decreased pain in many types of cancer (Azemar et al., 2007). Reduction of pectin Mw was crucial for its anti-cancer properties, therefore commercial pectin preparations that are currently available on the market do not possess anti-cancer activity.

In addition to anti-cancer activity, pectins have been shown to aid heavy metal detoxification. It has been demonstrated that the consumption of modified citrus pectin by Chinese children, hospitalized for toxic levels of lead resulted in decreased levels of lead in the blood and increased urinary excretion of lead (Zhao et al., 2008). Additionally, urinary excretion of toxic metals was not accompanied by the release of essential minerals (Eliaz et al., 2006). The presence of RGII in the modified citrus pectin was believed to be responsible for the selective chelation of toxic metals in the body. Therefore, pectin can be a safe heavy metal chelating agent, especially for children, who are most vulnerable to the long-lasting effects of heavy metal exposure.

Pectins have also shown beneficial bacterial adhesion properties. HG pectin domains have been reported to prevent adhesion of pathogenic strains of *E. coli* to intestinal uroepithelial cells. Dimers and trimers of galacturonic acid were the best pathogen blocking agents (Guggenbichler et al., 1997). Furthermore, pectins and pectic-oligosaccharides, derived using enzymatic hydrolysis, were able to inhibit Shiga-like toxins produced by pathogenic *E. coli* O157:H7 (Olano-Martin et al., 2003b).

Even though the abovementioned studies contain promising results in relation to the medical applications of pectin, better understanding of the structure-function relationships of pectins is required to explain the bioactive effects of pectin (and derived oligomers), which may be difficult due to their complexity and heterogeneity.

1.6 Pectin production

Pectin can be released from raw materials by water, chelating agents, alkalis, acids and enzymatic catalysis. Pectins extracted in different conditions and from different sources will differ not only in the degree of esterification, but also in GalA and neutral sugar contents, as well as in Mw.

Citrus peel and apple pomace, by-products from juice manufacturing, are the most commonly used raw materials for pectin production. Sugar beet pulp is used to a lesser extent, due to the high acetyl content of the pectin and the resulting decreased gelling ability (Rolin et al., 1998).

1.6.1 Traditional methods of pectin production used in industry

There are many naturally occurring endogenous and exogenous enzymes that will immediately attack pectin when fruit is being processed. Therefore, it is essential to either liberate pectin as soon as possible from the fresh raw material, or to stabilize the pomace or pulp by drying, which allows it to be stored or transported long distances. Before drying, the material is washed to remove sugars and acids (Rolin et al., 1998).

As most of the water-soluble pectin is extracted during juice manufacture, the pectin left in citrus peels or apple pomace is in an insoluble form. The current pectin production method employs high temperature acidic hydrolysis (70-90°C) with nitric, hydrochloric or sulfuric acid at pH between 1.5 and 2.5, conducted for several hours. The acid hydrolysis conditions cause partial depolymerization of pectin chain and other cell wall polymers. The low pH breaks ionic bonds holding the pectin in the plant tissue and the polymer is released into the liquid phase. Additionally, ester bonds are broken and pectin loses a proportion of its methyl and acetyl groups. Neutral sugar chains are also degraded. The pectin yield of the process increases with temperature, acidity and extraction time, but the length of the polymer decreases when more 'harsh' process conditions are applied and the product may lose some of its functional properties (Rolin et al., 1998; Voragen et al. 1995).

Next, the liquid containing pectin is separated from solids by filtration and/or centrifugation in one or more stages to remove the remaining plant tissue. The pectin extract is usually filtered hot to reduce its viscosity. The remaining plant material is typically used as cattle feed (Rolin et al., 1998). To avoid further degradation of the polymer by the acidic conditions, the pH of the pectin solution is increased to 3-4. The clarified extract is then concentrated by vacuum evaporators to 3-4% pectin content in order to minimize the volume of alcohol needed for precipitation (Voragen et al., 1995). Pectin can then be optionally deesterified or amidated by acid or alkali treatment. In fact, deesterification or amidation can be

conducted on clarified pectin extracts, concentrated pectin extracts or during pectin precipitation in alcohol.

Precipitation of pectin is carried out in alcohol. The precipitate is then washed in alcohol and pressed to remove contaminants such as sugars, pigments, polyphenols and other alcohol-soluble material. The alcohol is recovered by distillation. After the precipitated pectin is washed, it is dried and ground into a powder. The final product is tested, before it is blended with sugar (standardized) in order to obtain a product suitable for the final application. Standardization is performed with respect to a few properties, which are measured in a system imitating the application (Rolin et al., 1998).

The main disadvantage of this technology, raising environmental concerns, is generation of large volumes of acidic effluent that requires further treatment before release. Moreover, harsh acidic treatment causes depolymerisation and deesterification of the pectin chain.

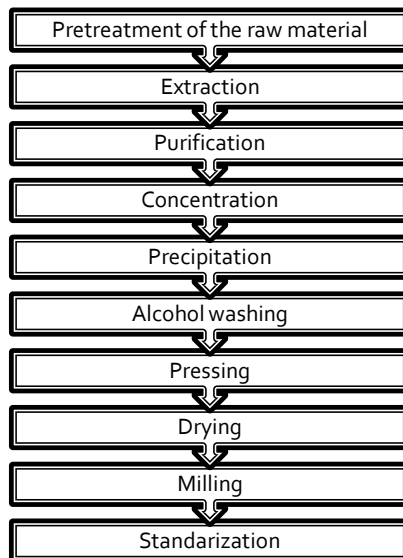


Figure 12. HM pectin production process.

1.6.2 Enzymatic extraction

Pectin can also be extracted enzymatically. Enzymatic extraction seems more advantageous in terms of energy consumption and waste management than acidic extraction. The process is usually carried out at pH between 3-5 and temperatures of around 50°C, which is more advantageous in terms of economy and environmental impact. Moreover, as the pH of the process is much higher than in the case of traditional acid-based approach, there is no need for waste neutralization. There are two among researchers working on the enzymatic pectin extraction process - the first one involves using HG and RG degrading enzymes, whereas the other employs cellulases and proteinases for degradation of other cell wall components. When using HG and RG degrading enzymes, caution has to be taken, because the degree of pectin polymerization will decrease as the reaction proceeds. In the other approach, involving proteinases and cellulases, the presence of any pectinolytic activities should be avoided to release the pectin in an intact form.

There have been several studies on enzymatic extraction of pectins from different plant sources described in literature. Thibault et al. (1998) extracted pectins from citrus, apple and sugarbeet pulps with endo-arabinase and endo-galactanase in conditions minimizing the pectate lyase side activity. They reported a relatively low pectin yield, namely 10.8% in the case of citrus pectin, and its Mw was highly reduced (47 kDa). These two enzymes were therefore found to be unsuitable for extraction of pectin on a commercial scale.

Zykwinska et al. (2008) extracted pectin using proteases and cellulases from a variety of substrates, including chicory roots, citrus peel, cauliflower florets and leaves, and sugar beet pulps. HM pectins (DE=68%) of high Mws and high GalA content (75%) were extracted in yields reaching 12.6% in the case of citrus peel. The authors noticed that higher pectin yields were obtained with enzymes possessing pectinolytic activities. Moreover, the characteristics of the extracted pectins were dependent on both the main enzyme activity and contaminant activities present. The extracted pectins possessed gelling abilities - HM citrus pectins gelled with sugar at low pH, whereas their corresponding LM pectin forms gelled with calcium (Zykwinska et al., 2009).

Ptichkina et al. (2008) tried extraction of pectin from pumpkin pulp, using an *Aspergillus awamori* enzyme preparation with cellulolytic and pectin esterase activities. Due to the presence of esterase in the preparation, the degree of esterification could be modified. The resulting pectins formed gels with 60% w/w sucrose at pH 3.

Another attempt to extract pectin involved the extraction of pectin with Celluclast 1.5L from kiwifruit (Yuliarti et al., 2011). Enzyme concentration had an effect on pectin yield and properties, such as viscosity and Mw distribution. Medium enzyme concentration resulted in highest pectin yield, viscosity and average-weight Mw, whereas at high enzyme concentration pectin was degraded, possibly due to the pectinolytic side activities of Celluclast 1.5L.

In conclusion, enzymatic methods of extraction of pectins present interesting alternatives to traditional acid hydrolysis technology. However, problems such as low pectin yields and reduced Mws are associated with enzymatic pectin extraction. Nevertheless, some enzymatically extracted pectins possessed functional properties (gelling), which makes enzymatic extraction a promising option that requires less energy input and no waste neutralization.

2. Enzymatic catalysis for efficient release of pectin with functional properties.

2.1Hypotheses and objectives

Lime peel, a by-product from the food industry, contains large amounts of pectin, which is currently extracted in the industry by acid hydrolysis. To our knowledge, there has been no successful attempt to extract lime pectin enzymatically and reach the yields and functionality that is currently obtained by acid technology. In this study we attempt to extract pectin in high yields and check whether the product possesses some functionality that could make it an alternative to products obtained in a traditional way.

2.2Key points

Dicotyledonous plants, including lime, contain typically 15-45% of pectic substances, 30-60% cellulose, 15-25% hemicelluloses and 10-15% glycoproteins in their primary cell walls on a dry basis (Selvendran, 1983). In this study, pectin was extracted using commercial cellulases, in order to degrade cellulosic and hemicellulosic substances in the peel, but liberate pectin in an intact form. Released pectins were characterized chemically and their functionality was assessed by US-SAG method, which evaluates gelling ability (Cox & Higby, 1944), as well as by testing them in a milk system for casein stabilization.

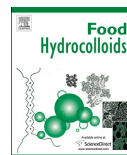
2.3 Experimental considerations

Six commercial cellulases were chosen for pectin extraction based on the previous experiments (unpublished data). Screening was conducted in pH from 3 to 4.8 to assure stability of the pectin polymer. In pH below 3 the enzymes would not be sufficiently active and the pectic polymer would be prone to acid hydrolysis. In pH above 5, and even at room temperature, pectin undergoes β -elimination reaction that reduces the chain length. The reaction is accelerated when the solution is heated. Afterwards, four small-scale extractions were chosen for a scale-up, based on pectin yields and their molecular weights. Molecular weights of pectins were measured by two methods: viscometry and GPC coupled to light scattering. As shown before, light scattering detection is very sensitive to the presence of aggregates, which are always present in pectin solutions. Even a small amount of such aggregates will have a dramatic effect on apparent molecular weight measured with light scattering, but almost no effect on viscosity. Therefore molecular weights measured by GPC coupled to light scattering were at least two times higher than those measured by viscometry.

Gelling ability was assessed by measuring °USA-SAG. All enzyme-extracted pectins were gelling, despite their molecular weights and high amount of neutral sugars. The reason for such a good gelling performance was probably very high DE of all pectin samples (around 80%) that allowed for hydrophobic interactions (as shown in Figure 2b). Additionally, pectins extracted with Validase TRL, Multifect B and GC 880 were also stabilizing acidified milk drinks. In this test, one has to assume a certain concentration of pectin in the drink before preparation, and adjust the dose based on the result. This is why stability tests were made with different doses of acid and enzyme-extracted pectins.

2.4Conclusions

Application of enzymes for the extraction of pectin enabled the release of pectic polymers with yields and functionality similar to the ones obtained in a traditional way. Commercial cellulases were very efficient extractants, which allowed for lower process temperature and higher pH as compared with the currently applied acid-based extraction technology. Even though the structure of enzyme-extracted pectin was different from acid extracts, they worked well in application.



Application of enzymes for efficient extraction, modification, and development of functional properties of lime pectin



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ABSTRACT

The objective of the present study was to transform “Waste to Food” using enzymes to recover value-added food ingredients from biomass. Six commercial cellulases were screened to generate proof of concept that enzymes are selective and efficient catalysts for opening of lime peel biomass to recover pectin. The most efficient enzyme preparation was Laminex C2K derived from *Penicillium funiculosum* which, during 4 h treatment at pH 3.5, 50 °C, released pectin with similar yield (23% w/w), molecular weight (69 kDa), and functional properties e.g. gelling, stabilization of acidified milk drinks and viscosity as the classically acid-extracted pectins (8 h treatment at 70 °C, pH < 2). Carbohydrate microarray analysis showed that enzymatically extracted pectin mainly contained highly methylated pectin (chemical compositional analysis indicated degree of esterification up to 82%), whereas acidically extracted pectins were more heterogeneous with regard to degree of esterification and had lower degrees of esterification (67–74%). A high degree of esterification in enzymatically extracted pectin may be directly exploited commercially as the so-called Ultra-Rapid-Set pectin, which gels particularly fast at higher temperatures. The Laminex CK2 extracted pectin polymers were not sensitive to the presence of Ca²⁺ ions, they formed a gel at low pH in the presence of sugar and were able to stabilize acidified milk drinks. Further modification by enzymatic de-esterification of the pectin extracted with Laminex C2K improved its calcium sensitivity and ability to stabilize acidified milk drinks. The present study demonstrates that it is possible to substitute classical acid-based extraction by enzymatic catalysis and obtain pectin products with desirable functional properties.

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1. Introduction

Refinery of biomass to recover labile value-added components is an important future requirement to upgrade many million tons of side-streams from the agricultural and dairy industries to important food ingredients. At present most of the processes using biomass are exclusively focused on total hydrolysis of the polymers to form fermentable monosaccharides for bio-ethanol production. The commercial enzymes are able to catalyse the degradation of most of the plant cell wall materials including the

hydrocolloids, but there has so far been very little incentive to generate selective processes with several product lines, where value-added labile products could be isolated before the residual biomass would be used for bioethanol. It is, however, not a simple task to integrate an up-stream process, where labile polymers such as hydrocolloids are to be harvested before degradation of the residual biomass to monosaccharides. There is a need for improved enzymatic processes, integrating hydrocolloid extraction with side-stream and biofuel production.

The plant cell wall is very complex and in dicotyledonous plants it is comprised typically of 15–45% of pectic substances, 30–60% cellulose, 15–25% hemicelluloses and 10–15% glycoproteins on a dry basis (Selvendran, 1983). Pectin is mainly composed

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of three structural domains: homogalacturonan (HG), type I rhamnogalacturonan (RG I) and type II rhamnogalacturonan (RG II) (Verheerdtbruggen & Knox, 2006). HG is the dominant type of pectin in lime peel and consists of a linear chain of α -(1 \rightarrow 4)-linked D-galacturonic acid (GalA) residues, being partly methyl-esterified at O-6 and sometimes acetyl-esterified at O-2 or O-3 (Voragen, Beldman, & Schols, 2001). RG I, composed of repeating disaccharide units: (1 \rightarrow 2)-L-Rha-(1 \rightarrow 4)-D-GalA (where Rha denotes rhamnose) is abundantly substituted by arabinan and galactan side chains at the O-4 position of Rha (Schols & Voragen, 1994; Yapo, Lerouge, Thibault, & Ralet, 2007). RG II, the least abundant domain, is composed of a linear backbone chain of D-GalA, substituted with L-Rha, D-Gal (galactose) and many unusual sugars such as apiose, 3-O-methyl-L-fucose, 2-O-methyl-D-xylose, 3-C-carboxy-5-deoxy-L-xylose, 3-deoxy-D-manno-octulosonic acid and 3-deoxy-D-lyxo-heptulosaric acid (Voragen et al., 2001).

Due to its functional properties, pectin is used in the food industry as an ingredient - it enables gelling, provides viscosity, stabilizes proteins and acts as a fat mimetic (Voragen et al., 2001; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995; Willats, Knox, & Mikkelsen, 2006). The current extraction methods employ high temperature acidic hydrolysis with nitric or sulphuric acid at pH between 1.5 and 2.5. The main disadvantage of this technology, raising environmental concerns, is the generation of large volumes of acidic effluent that requires further treatment before release. Moreover, harsh acidic treatment may give rise to undesirable breakdown of the pectin chain. Enzymatic extraction thus seems more advantageous in terms of selectivity, energy consumption and waste management.

The hypothesis behind this study was that special enzymes can catalyse release of pectin with yield, viscosity and functional properties similar to that of acidic extraction. Many enzyme activities, including cellulases and hemicellulases, are necessary to release pectin from the cell walls. In this paper the effects of four commercial cellulases were compared on the basis of pectin yields and functionality. Commercial enzymes were chosen because their production is already optimized, safe and economical and therefore their implementation in the production of pectin would be more straightforward than in case of newly designed enzymes. The present study demonstrated that lime pectin can be enzymatically extracted at high yield and that it possesses gelling and stabilizing properties with similar performance to acid extracted pectin from lime fruits.

2. Methods

2.1. Peel and enzymes

Dry lime peel was obtained from the DuPont Pectin Plant (DuPont Nutrition and Biosciences Mexicana S.R.L., Tecomán, Colima, Mexico). The pretreatment involved milling the peel to pass a 35-mesh size screen (centrifugal mill Retsch ZM 200, Haan, Germany). The enzymes (Table 1) Laminex C2K, Multifect B, GC220 and GC880 were supplied by DuPont Industrial Biosciences (Leiden, The Netherlands). Validase TRL was purchased from DSM (South Bend, IN) and Cellulase from *Trichoderma reesei* ATCC 26921 was kindly provided by Sigma–Aldrich (Steinheim, Germany). The overview of the six enzymes, including their sources, working conditions and activities (given by the manufacturer) is presented in Table 1. Enzymes used for the de-esterification of pectin, Rapidase FP Super and Collupulin Liq, were obtained from DSM (South Bend, IN). Endo- and exo-polygalacturonases, used for enzymatic fingerprinting, were obtained and purified as described in Limberg et al. (2000b).

2.2. Chemicals

Avicel PH 101, D-(+)-galacturonic acid monohydrate, polygalacturonic acid from orange, potassium sodium tartrate tetrahydrate and 3-hydroxybiphenyl were purchased from Sigma–Aldrich (Steinheim, Germany). Disodium tetraborate-10-hydrate, sodium acetate trihydrate, sodium sulphate, sodium arsenate dibasic heptahydrate, sodium carbonate and copper (II) sulphate pentahydrate were purchased from Merck (Darmstadt, Germany). Ammonium molybdate tetrahydrate and sodium bicarbonate were purchased from Riedel-de Haën (Seelze, Germany). PD-10 Desalting columns were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Sodium hexametaphosphate and tri-sodium citrate dihydrate were purchased from VWR International (Poole, England). Glucono- δ -lactone was purchased from SFK Food A/S (Viborg, Denmark). Milex 240 skimmed milk powder was purchased from Arla Food Ingredients (Viby J, Denmark). Pectins E81, F31 and F19 were provided by DuPont Nutrition and Biosciences (Brabrand, Denmark).

2.3. Determination of enzyme activities

The six enzyme preparations were analysed for the presence of cellulase, polygalacturonase, pectin lyase and rhamnogalacturonan I lyase activities. Cellulase was measured at 50 °C in 50 mM acetate buffer, pH 4.8 containing 10% Avicel PH 101 as the substrate. Initial reaction rates were measured by quantification of reducing sugars released according to Lever (1972). The absorbance was measured at 410 nm in an Infinite 200 microplate reader (Tecan, Salzburg, Austria) according to Thomassen, Vignæs, Licht, Mikkelsen, and Meyer (2011). Polygalacturonase was determined at 50 °C in 50 mM acetate buffer, pH 4.8 using 0.1 M polygalacturonic acid as the substrate. Initial reaction rates were quantified by measuring the amount of reducing sugars released by Nelson and Somogyi method (Nelson, 1944; Somogyi, 1945), at 620 nm using a SpectraMax 19 microplate reader (Molecular Devices, Sunnyvale, CA). Pectin lyase and RGI lyase were measured according to Silva, Larsen, Meyer, and Mikkelsen (2011). Substrates for enzymatic reactions were highly methyl esterified citrus pectin (DuPont, Brabrand, Denmark) and RGI from potato (Megazyme, Bray Business Park, Ireland) respectively. The activity measurement was based on the formation of a double bond (unsaturated galacturonic acid) having a strong absorbance at 235 nm. Before the assay, the enzymes were partly purified using PD-10 desalting Columns, to remove UV impurities. The reactions were carried out in Infinite 200 microplate reader at 40 °C.

2.4. Small scale enzymatic extraction protocol

1.35 g of milled lime peel was mixed with 40 ml of 50 mM citric acid buffer at a specified pH value (3, 3.5, 4 or 4.8). The mixture was heated to 50 °C before addition of 75 μ l of the enzyme. After addition, the sample was incubated in a water bath for 4 h at 190 rpm. The reaction was terminated by heating at 100 °C for 5 min to inactivate the enzyme. The released pectin polymer in solution was separated from the residual biomass by centrifugation for 10 min at 3500 rpm (Varifuge 3.0, Heraeus Sepatech, Osterode, Germany). The pectin solution was decanted off and the remaining biomass was mixed with 20 ml of water and incubated for an hour at 50 °C and 190 rpm to recover residual pectin. After centrifugation the pectin solution was combined with the former pectin solution and the resulting mixture was filtered through Celite 545 (Celite France S.A., Nanterre Cedex, France). Pectin was precipitated by mixing with a double volume of propan-2-ol, followed by agitation for 1 h. The precipitated pectin was separated by filtration through a cotton canvas and dried overnight in a 40 °C ventilated oven.

Table 1

The sources, working conditions and activities of six commercial cellulases used in the study. Data taken from the enzyme specification sheets provided by the manufacturers.

	Description	Source	Working conditions (optimum)		
			pH	Temperature (°C)	Activity
GC220	Cellulase, hemicellulase (β-glucanase)	<i>T. reesei</i>	3.5–6.5	50–70	6200 CMCU/g ^a
GC880	Cellulase		4.5–6.5 (4.5–5.0)	40–68 (50–65)	10,300 CMCU/g
Laminex C2K	Cellulase, hemicellulase (xylanase, arabinoxylanase)	<i>P. funiculosus</i>	3.5–6.5 (5.0)	45–65 (55)	3150 CMC-DNSU/g ^b
Validase TRL	Cellulase, hemicellulase (xylanase, mannanase, β-glucanase)	<i>T. longibrachiatum</i>	3.5–6.5 (4.0–5.0)	50–60 (50–55)	6800 CU/g ^c
Multifect B	Cellulase, hemicellulase (β-glucanase)	<i>T. reesei</i>	4.0–6.0 (5.0)	40–60 (50)	2250 BGLU/ml ^d
Cellulase from <i>T. reesei</i>	Cellulase	<i>T. reesei</i>	4.5–6.0	50–60	1500 NCU/g ^e

^a Carboxymethylcellulose units.

^b Carboxymethylcellulose-dinitrosalicylic acid units.

^c Cellulase units.

^d Beta-glucanase units.

^e Novo cellulase units.

Dried pectin was ground to <0.5 mm in an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). Pectin yield (% w/w) was calculated as a weight ratio of the dried pectin to the starting material (milled lime peel), both expressed on dry matter basis.

2.5. Enzymatic extraction protocol for 3 L scale

Up-scaling to 3 L volume was performed using a 3-neck round bottom flask with agitator inside, connected to a titration device Titrino 877 Plus (Metrohm, Herisau, Switzerland) to maintain the reaction pH constant by addition of 0.1 M NaOH. The pulp: water ratio was the same as in the small scale extraction. The experiment was performed as described for the small scale process except for the following modification: before precipitation the pectin solution was concentrated using a rotary evaporator.

2.6. Acidic extraction protocol for 3 L scale

The experiment was performed similarly to the protocol for enzymatic extraction for 3 L scale with minor modifications. Briefly, 100 g of lime peel was mixed with 3 L deionized water and heated to 70 °C. Next, the pH was adjusted to 1.7 (extraction A) or 2.0 (extraction B) with 40% nitric acid and the mixture was incubated in a 3-neck round bottom flask with agitator inside for 8 h (extraction A) or 4 h (extraction B). The released pectin polymer was separated from the residual biomass by centrifugation for 10 min at 3500 rpm (Varifuge 3.0, Heraeus Sepatech, Osterode, Germany). Pectin was decanted off and the remaining biomass was mixed with 2 L of water and incubated for an hour. After centrifugation the pectin was combined with the former pectin solution and the resulting mixture was filtered through Celite 545 (Celite France S.A., Nanterre Cedex, France). The solution was concentrated using a rotary evaporator. Precipitation, drying and grinding was performed as described in the protocol for small scale enzymatic extractions.

2.7. Enzymatic de-esterification of pectin with Rapidase FP Super

25 g of enzymatically extracted pectin with Laminex C2K at pH 3.5 (described in Section 2.5) was dissolved in 1 L of hot deionized water under efficient agitation. The pH was adjusted to 4.0 and 10 μl of enzyme Rapidase FP Super was added. The temperature of de-esterification was maintained at 50 °C and the pH was kept at 4.0 by automatic addition of 1 M NaOH until the desired DE was

achieved (estimated from consumption of 1 M NaOH). The enzyme was inactivated by boiling the solution for 5 min and the pectin was precipitated, dried and milled as described in Section 2.4.

2.8. Enzymatic de-esterification of pectin with Collupulin Liq

25 g of enzymatically extracted pectin with Laminex C2K at pH 3.5 (described in Section 2.5) was dissolved in 1 L of hot deionized water under efficient agitation. The pH was adjusted to 6.0 and 10 μl of enzyme Collupulin Liq was added. The temperature of de-esterification was maintained at 40 °C and the pH was kept at 6.0 by automatic addition of 1 M NaOH until the desired degree of esterification was achieved (estimated from consumption of 1 M NaOH). Next, the pH was decreased to 3.0 by addition of 42% HNO₃. The enzyme was inactivated by boiling the solution for 5 min and the pectin was precipitated, dried and milled as described in Section 2.4.

2.9. Determination of the degree of esterification

Degree of pectin esterification was determined according to the method described in the Food Chemical Codex (3rd ed.) (1981, p. 216). Briefly, pectin samples were washed in 60% propan-2-ol containing 5% HCl, followed by washing in 60% and 100% propan-2-ol. Next, 0.2 g of washed and dried material was dissolved in 100 ml deionized water and the sample was titrated with 0.1 M NaOH using phenolphthalein as an indicator (the volume of 0.1 M NaOH was recorded as V₁). Then the sample was saponified by addition of 10 ml 1 M NaOH, followed by mixing for 15 min. Next, 10 ml of 1 M HCL was added to the sample and the mixture was again titrated with 0.1 M NaOH until colour change (volume V₂). The DE was calculated according to the formula:

$$DE = \frac{V_2}{V_1 + V_2} \times 100\%$$

2.10. Determination of galacturonic acid in pectin

Galacturonic acid content was determined by the colourimetric m-hydroxybiphenyl method (Blumenkrantz & Asboe-Hansen, 1973). The measurements were performed in a microtiter plate, according to van den Hoogen et al. (1998), using Tim-200 plate reader equipped with 492 nm filter (Teknunc A/S, Roskilde, Denmark).

2.11. Determination of the total galacturonic acid content in the lime peel

The content of galacturonic acid in the milled lime peel was measured according to Ahmed and Labavitch (1978). Briefly, 5 mg of the milled lime peel was hydrolysed in 3 ml of chilled concentrated H_2SO_4 . Next, the galacturonic acid content of the hydrolysate was measured as described in Section 2.10. Polygalacturonic acid from orange was used as a control sample during acid hydrolysis and the measurement of galacturonic acid content.

2.12. Determination of the viscosity–average molecular weight

The viscosity–average molecular weights (M_w) of pectin samples were determined using a H  ppler viscometer, type 002-7580 (Haake, Karlsruhe, Germany) according to Limberg et al. (2000a). Briefly, the duration of the fall periods of the ball in a 1% sodium hexametaphosphate solution (pH 4.5) with and without 0.09% pectin were determined. The viscosity–average molecular weight was calculated according to the formula:

$$M_w = \left(\frac{T_{\text{test}}}{T_{\text{ref}}} - 1 \right) \times 200,000$$

Where T_{test} was the average time of the fall of the ball in pectin solution and T_{ref} was the average time of the fall of the ball in the sodium hexametaphosphate solution.

2.13. Determination of molecular weight by gel permeation chromatography (GPC)

Three GPC SUPREMA-LUX columns – 3000  , 10,000   and 30,000   (PSS, Mainz, Germany) were used for gel permeation chromatography. Pectin solutions (1 g/L) were filtered through a 0.45  m filter before injection. The mobile phase was 0.05 M LiNO_3 with 0.02% NaN_3 which was filtered through a 0.22  m filter before use. Separations were carried out at 40  C with a flow rate of 0.6 ml/min. The eluent was monitored by a MALLS detector DAWN EOS (Wyatt Technology Corporation, Santa Barbara, CA) and a serial connected refractive index detector Optilab reX (Wyatt Technology Corporation). The data were analysed using Astra 5 software (Wyatt Technology Corporation) and a value of 0.131 was used for the refractive index increment (Corredig, Kerr, & Wicker, 2000).

2.14. Neutral sugar composition

After hydrolysis of pectin in 1 M sulphuric acid, the neutral sugars were detected by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). Before hydrolysis, the pectin sample was washed three times in 80% ethanol followed by washing one time in 96% ethanol to remove sugars. The hydrolysis in 1 M sulphuric acid was carried out at 100  C for 6 h. After hydrolysis the sample was diluted and filtered through a 0.45  m filter. The content of D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose and D-xylose in pectin was determined. The separation and quantification was performed by HPAEC-PAD using an ICS-3000 system (Dionex Corp., Sunnyvale, CA).

The columns used were CarboPacTM PA1 (4 mm   250 mm) analytical column and a CarboPacTM PA1 guard column. Eluent A consisted of deionised water and eluent B of 150 mM NaOH. 20 min isocratic elution with 15 mM NaOH was followed by 10 min washing with 150 mM NaOH. The flow was kept at 1 ml/min. Before injection of each sample (50  l) the column was reequilibrated with

15 mM NaOH for 8 min. Analysis was made with the program Chromeleon 6.80 software (Dionex Corp., Sunnyvale, CA).

2.15. Characterization by carbohydrate microarrays

Enzymatic and acid extracted pectins as well as three standard lime pectin samples were dissolved in deionized water to a concentration of 4 mg/ml. Three replicates per sample were prepared. Each replicate was printed as a two-fold dilution followed by 3 five-fold dilutions. Each dilution was printed in duplicate, resulting in a total of 24 spots per sample. All dilutions were performed in printing buffer (55.2% glycerol, 44% water, 0.8% Triton X-100). Samples were printed using a piezoelectric microarray robot (Sprint, Arrayjet, Roslin, UK) on nitrocellulose membrane with a pore size of 0.45  m (Whatman, Maidstone, UK). Standard pectin samples included the commercial pectin E81 from lime peel with a DE of 81% and pectins F31 and F19 with a DE of 31% and 19% respectively, which are derived from E81 treated with pectin methyl esterase from *Aspergillus niger*. The three samples (DuPont Nutrition Biosciences, Brabrand, Denmark) were described in Limberg et al. (2000a).

Nitrocellulose microarrays were probed as described in Pedersen et al. (2012). Briefly, arrays were probed with a collection of anti-pectin monoclonal antibodies from PlantProbes (Leeds, UK) 1/10 diluted in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.7 mM KH_2PO_4 , pH 7.5), containing 5% w/v low fat milk powder (MPBS) for 2 h. Once washed with PBS, microarrays were incubated with an anti-rat secondary antibody conjugated to alkaline phosphatase (Sigma, Poole, UK) 1/5000 diluted in MPBS for 2 h. Developed microarrays were scanned at 2400 dpi (CanoScan 8800F, S  borg, Denmark) and converted to TIFFs. Antibody signals were quantified using appropriate microarray software (Array-Pro Analyzer 6.3, Media Cybernetics, Rockville, USA). Data was presented as a heatmap, where the maximal mean spot signal value was set to 100 per each antibody and all other values were normalized accordingly. Colour intensities are correlated to mean spot signal. A cut off of 5 was applied.

2.16. Enzymatic fingerprinting

Enzymatic fingerprinting was performed according to Limberg et al. (2000b). One ml of pectin solution (5 mg/ml in 50 mM of sodium acetate buffer, pH 4.5) was incubated with 0.2 U of endo-polygalacturonase for 24 h at 40  C. The reaction was stopped by boiling the samples for 5 min. After cooling, 0.1 U of exo-polygalacturonase was added to samples and the reaction proceeded for another 24 h at 40  C, followed by enzyme inactivation. 1 mg/ml glucuronic acid was used as an internal standard.

Galacturonic acid content of samples was determined using HPAEC-PAD with ICS-3000 system (Dionex Corp., Sunnyvale, CA). The columns used were CarboPacTM PA10 (2 mm   250 mm) analytical column and a CarboPacTM PA10 guard column. Eluent A consisted of 100 mM NaOH and eluent B of 100 mM NaOH with 1 M sodium acetate. The flow was kept at 0.15 ml/min. Elution gradient: 90% A and 10% B at 0 min linearly changing to 50% A and 50% B at 25 min. The column was rinsed with 100% eluent B for 5 min before equilibration with starting conditions for 10 min was executed.

2.17. Stabilization of milk drinks

Milex 240 skimmed milk powder was dissolved in water and acidified to pH 4.0 with glucono- -lactone. The solution was combined with pectin to mimic a drinking yogurt with 8% milk solid non-fat. After homogenization at 20 MPa with APV-1000 homogenizer (APV, S  borg, Denmark), it was pasteurized at 75  C for

10 min and stored overnight at 4 °C. Next day the sample was centrifuged at 2800 g for 20 min and the weight of the wet sediment was recorded and expressed in % of the total weight of the sample.

2.18. Gelling ability

Determination of the gelling ability of pectin was performed according to US-SAG method (Cox & Higby, 1944). Briefly, a pectin gel containing 65% soluble solids (pH 2.2) was prepared in a glass and stored for 24 h at 25 °C. Next day, the gel was removed from the glass and the decrease of its height within 2 min was measured with a ridgeline. The results were expressed in °US-SAG.

2.19. Calcium sensitivity

Calcium sensitivity was measured according to Limberg et al. (2000a). Briefly, two 290 g portions of 0.6% pectin solution (pH 1.5) were prepared, followed by addition of 10 ml of 0.25 M CaCl₂ to the first portion (test sample) and 10 ml of 1% sodium hexametaphosphate to the second portion (reference sample). Next, 50 ml of 1 M NaOAc buffer (pH 4.7) was added to both samples and they were stirred for 15 min. Viscosities of the test and reference samples were measured using Brookfield DV-II viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, MA), spindle 2, at 60 rpm and 21 °C. Calcium sensitivity was calculated as:

$$CS = \frac{\text{viscosity of the test sample}}{\text{viscosity of the reference sample}} - 1$$

2.20. Data analysis

One-way analysis of variance (ANOVA) and post hoc Tukey tests were performed using IBM SPSS Statistics 21.0.0 (New York, USA).

3. Results

3.1. Small-scale screening experiment at different pH

The major activity of the six enzymes used in the study was cellulase, whereas low activities of polygalacturonase, pectin lyase and rhamnogalacturonan I lyase were also observed (Table 2). Since pectin is known to be unstable at pH above 5 when exposed to elevated temperatures (Voragen et al., 2001), it was decided to test enzymes performance at pH between 3.0 and 4.8, at a temperature of 50 °C. These experimental conditions were suitable for all enzymes, as indicated in Table 1. Since all six commercial enzymes contained various cell-wall degrading activities (as indicated in Table 1), it was decided to add the same enzyme volume to each extraction tube.

The yields and viscosity–average molecular weights (M_w) of enzymatically extracted pectin at pH 3, 3.5 and 4 using GC220 (A),

cellulose from *T. reesei* (B), GC880 (C) or MultifectB (D) were at a low level (Fig. 1). In contrast the last two enzymes, Laminex C2K (E) and Validase TRL (F), gave very high yields (19–31%) and relatively high viscosity–average M_w . At pH 4.8 pectin yields obtained by all enzymes were very high (24–32%), except for that of GC220 where only 15% yield was observed. A high yield and a high viscosity–average M_w of the pectin polymer are prerequisites for the economy of the process and good pectin quality. The best solution for a high extraction recovery (>25%) and a large M_w (approx. 60 kDa) was achieved at pH 3.5 by the use of Laminex C2K (E) and Validase TRL (F).

Compositional analysis of the monomeric building blocks confirmed that pectin samples extracted by the six commercial enzyme preparations were a genuine polymer composed mainly of galacturonic residues (71–85% w/w), which was higher than the 65% legal boundary level, specified by FAO, FCC and EU on the acid washed pectin.

3.2. 3-L extraction scale

Based on the results obtained in the screening experiments, four enzymes (GC880, Multifect B, Laminex C2K and Validase TRL) were selected for a scale up to 3 L volume to validate the structure and functional properties of enzymatically extracted pectin samples. Due to decrease of pectins molecular weight with increasing pH, a value of 3.5 was chosen in the case of enzymes GC880, Laminex C2K and Validase TRL. At this pH value both yield and viscosity–average M_w were at high level. The screening experiment demonstrated that in the case of Multifect B molecular weight of pectin was preserved until pH 4, therefore this pH was chosen for a scale-up. Additionally, two classical acidic extractions using pH 1.7 and 2, both at 70 °C, were also performed on the same lime peels to compare the yields, molecular weights, composition and functionality of both the enzymatic and acidic extracted pectin samples.

3.2.1. Composition

The yields of enzymatically extracted pectin samples at 3 L scale were similar to their small scale equivalents in the screening phase (Table 3) ranging from 17.7% in the case of Multifect B to 26.3% for Validase TRL. The yields of two acid extracted pectin samples differed significantly – pectin A, extracted at lower pH for longer time, was released in almost double amount as compared to pectin B, extracted at milder conditions for shorter time. All enzyme extracted pectin samples had a very high degree of esterification, reaching 80%. Pectic polymers extracted with acid had lower degrees of esterification (74 and 67%), which could be attributed to hydrolysis of ester groups at the acidic extraction conditions.

Molecular weights of all pectin samples were measured by two methods: viscosimetry and GPC. Viscosity–average M_w of enzyme extracted pectin samples ranged from 57 to 91 kDa, which was lower than in the case of acid extracted pectin B (145 kDa). Viscosity–average M_w of acid extracted pectin A was only 63 kDa, indicating degradation of the polymer by the more harsh extraction conditions. GPC sorts the molecules by size, with the largest eluting first and the smallest last. The MALLS peak height is proportional to the product of concentration and hydrodynamic volume of the molecules, whereas RI peak increases with concentration (Wyatt, 1993). GPC profiles of enzyme extracted pectin samples (Fig. 2A) appeared more heterogeneous than acid extracted pectin samples (Fig. 2B), due to more diversified M_w and larger amounts of neutral sugars. MALLS signals were eluting at lower retention volume than RI signals, which suggested the presence of a high molecular weight component in low amount (low RI signal). In the case of acid extracts, presented in Fig. 2B,

Table 2

Evaluation of commercial enzyme preparates for cellulase and pectolytic activities (polygalacturonase, pectin lyase and rhamnogalacturonan I lyase). All activities were measured at pH 4.8.

Enzyme	Enzyme activities [U/ml]			
	Cellulase	Polygalacturonase	Pectin lyase	RG1 lyase
GC220	14,990	2.2	0.5	1.3
GC880	8681	0.3	4.4	5.7
Laminex C2K	4790	3.8	0.4	1.3
Validase TRL	5464	0.3	3.2	0.9
Multifect B	7652	0.1	0.5	1
Cellulase from <i>T. reesei</i>	7203	1.4	1.6	0.8

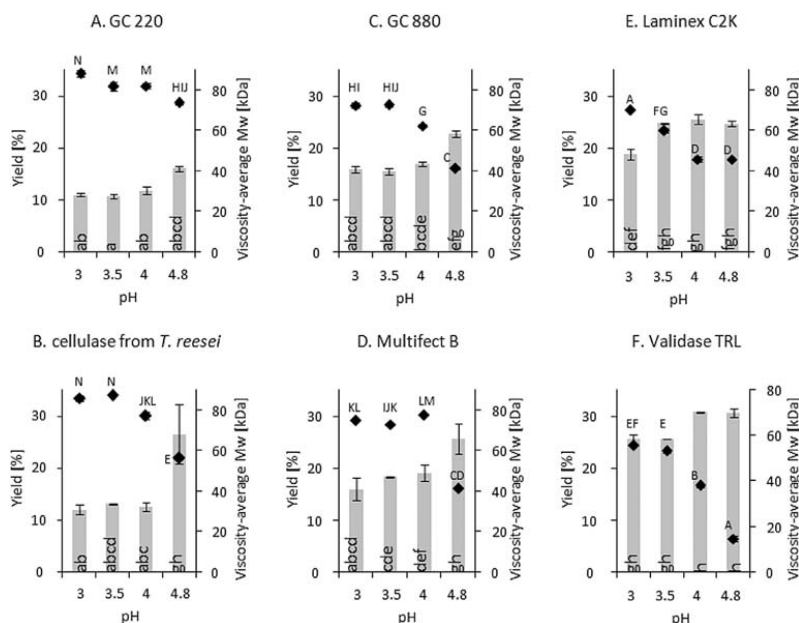


Fig. 1. Pectin yields (grey bars) and viscosity-average molecular weights (black points), obtained after 4 h digestion with GC220 (A), cellulase from *T. reesei* (B), GC880 (C), Multifect B (D), Laminex C2K (E) and Validase TRL (F) at pH 3, 3.5, 4 and 4.8. Error bars indicate standard deviation. Means that do not share a letter are significantly different ($p < 0.05$).

MALLS and RI peaks were more superimposed, which suggested lower heterogeneity of the polymers. The RI peak eluting between 35 and 37 ml, present in both Fig. 2A and B, was due to the presence of salt in pectin samples.

Weight-average molecular weights measured by GPC were 1.4–4 times higher than viscosity-average molecular weights measured by viscometry. There could be several reasons for this discrepancy. First of all, these two methods measured different polymer characteristics (hydrodynamic volume and viscosity), neither of them being the true molecular weight of the polymer. Moreover different solvents used in the two systems, could cause different expansion of the molecules or stimulate the formation of aggregate, which has been described in details by Fishman, Chau, Kolpak, and Brady (2001). Light scattering detection, used together with GPC, is known to be particularly sensitive to the presence of aggregates, resulting in molecular weights up to 1000 kDa (Jordan & Brant, 1978; Sorochan, Dzizenko, Bodin, & Ovdov, 1971). On the contrary, sodium hexametaphosphate present in

viscosity analysis eliminated the risk of viscosity increase, caused by the presence of residual calcium in the pectin sample.

Galacturonic acid content of enzyme extracted pectin samples was between 81 and 84% and they contained 12–15% neutral sugars (w/w), as presented in Table 4. In the case of acid extracted pectin samples galacturonic acid content was higher (90%), but neutral sugars reached only 9%.

3.2.2. Functionality

All pectins gelled at low pH and presence of 65% sugar, as expressed in °US-SAG (Table 5). Typically, commercial pectin is blended with sugar to obtain a gelling strength of 150 °US-SAG. High SAG value in raw pectin means that a low amount of pectin is needed to obtain a desired gelling strength of the blend. Moderate levels of US-SAG in case of enzyme extracted pectins were caused by pre-gelling, which occurred due to their very high DE level. Among enzyme extracted pectin samples best gelling was observed in the case of pectins extracted with Multifect B and GC880,

Table 3

Extraction conditions, pectin yields, degrees of esterification and molecular weights of enzyme and acid extracted pectin samples in 3 L scale. Molecular weights were measured by two methods: viscometry and gel permeation chromatography in-line with multi-angle light scattering detection. Values are means of 2 measurements \pm standard deviation. Values that do not share a letter within the column are significantly different ($p < 0.05$).

Enzyme/acid	Extraction conditions			Yield [%]	DE [%]	M_w [kDa]	
	Time [h]	Temp [°C]	pH			M_w^a	M_w^b
Laminex C2K	4	50	3.5	22.5	82.2 ± 2.5^c	69.2 ± 0.6^c	115.0 ± 1.4^a
Validase TRL	4	50	3.5	26.3	79.1 ± 0.8^c	56.6 ± 0.4^a	225.5 ± 9.2^b
Multifect B	4	50	4	17.7	79.3 ± 1.0^c	91.2 ± 0.6^c	201.5 ± 3.5^b
GC880	4	50	3.5	17.9	78.4 ± 1.4^{bc}	82.3 ± 0.6^d	198.0 ± 25.5^b
HNO ₃ (A)	8	70	1.7	25.6	67.3 ± 1.5^a	63.3 ± 0.5^b	110.7 ± 0.6^a
HNO ₃ (B)	4	70	2	13.6	73.9 ± 1.2^b	145.5 ± 5.5^f	194.5 ± 3.1^b

^a Viscosity-average molecular weight measured by viscometry.

^b Weight-average molecular weight measured by gel permeation chromatography in-line with multi-angle light scattering detection.

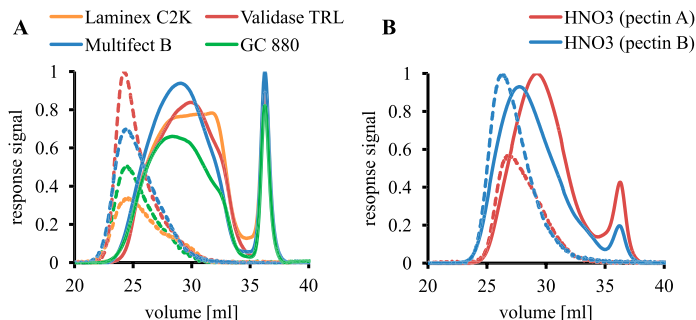


Fig. 2. GPC profiles of enzyme (A) and acid (B) extracted pectins. Dotted lines indicate MALLS signal (molecular weight and distribution detector), whereas continuous lines represent RI signal (concentration detector).

probably due to highest viscosity—average M_w . Acid extracted pectin B provided best gelling (226 °US-SAG) due to highest pectin viscosity—average M_w and low content of neutral sugars.

The ability of pectic polymers to stabilize milk drinks is presented in Fig. 3. Stabilization occurred due to electrostatic interactions between HG pectin domains and casein, and repulsion between charged pectic stretches, surrounding casein micelles (Tromp, de Kruif, van Eijk, & Rolin, 2004). Enzymatically extracted pectins were tested at 3 concentration levels, namely 0.2, 0.3 and 0.4% (w/w) (Fig. 3A). Such a broad concentration range was chosen, because it was difficult to predict the performance of the pectin in the milk drink. In the case of acid extracts, lower pectin levels were chosen (0.15, 0.2 and 0.25%) as presented in Fig. 3B. This more narrow concentration range was selected on the basis of the previous experience with acid extracted pectins, produced in similar conditions. Pectin samples extracted with Validase TRL, Multifect B and GC 880 were able to stabilize milk drinks, whereas Laminex C2K pectin did not prevent sedimentation even at highest concentration tested. Validase TRL pectin was stabilizing the drinks already at the 0.3% addition level, whereas Multifect B and GC880 pectins prevented sedimentation at 0.4% concentration. Acid extracted pectin A was not stabilizing the drinks at the three levels initially chosen and therefore it was tested at higher concentrations, where it provided satisfactory stability. Acid extracted pectin B was a very good stabilizer already at 0.15% level, probably due to it having the highest viscosity—average M_w . The enzymatically extracted pectin samples were not sensitive to calcium due to high DE (Table 5). This was in agreement with the model commercial pectin E81 (URS 1200, DuPont Nutrition Biosciences, Grindsted, Denmark), described in Limberg et al. (2000a), having a comparable degree of esterification (81%), but low calcium sensitivity. Polygalacturonase, present in enzyme preparations, could attack the glycosidic bonds between unmethylated galacturonic acid units, thus decreasing the calcium sensitivity of enzyme extracted pectin. Acid extracted pectin B had

much higher calcium sensitivity than the rest of the samples, due to much higher viscosity—average M_w , which was essential for creation of a strong pectin network in the gel.

The presence of stretches of unesterified galacturonic acid (block sequences) was assessed by enzymatic fingerprinting (Table 5). The blockwise distribution of unesterified galacturonic acid is essential for cross-linking with calcium molecules, even though the exact number of monomers forming this structure is unknown (Limberg et al., 2000b). Presence of block structures and their length are also important for the ability of pectin to stabilize milk drinks. In the case of too long blocks of free carboxyl groups, there are no sufficiently long dangling pectin chains, protruding into the liquid, repelling each other and thus increasing the stability of the dairy system (Syrbe, Bauer, & Klostermeyer, 1998). In an opposite case, too short free galacturonic acid fragments do not bind strongly to casein particles. The amount of galacturonic acid released by endopolygalacturonase indicates non-esterified galacturonic acid monomers in block sequence at the non-reducing end or inside the block sequence, whereas endo + exopolygalacturonase digestion release all non-esterified galacturonic acid monomers located between other non-esterified monomers (Limberg et al., 2000b). Endo + exo digestion of enzyme extracted pectin samples resulted in only between 1 and 1.5% GalA released, similarly to pectin E81, having comparable degree of esterification. Acid extracted pectin samples had more monomers located in block sequences. Digestion of polygalacturonic acid (PGA), added to Table 5 for comparison, resulted in release of over 57% of galacturonic acid (from Limberg et al., 2000b).

3.2.3. Characterization by carbohydrate microarrays

Enzyme and acid extracted pectins were also analysed using carbohydrate microarrays. Pectin samples were spotted as microarrays and information about the structural composition of the different samples was obtained by probing the arrays with five

Table 4

Chemical composition of enzyme and acid extracted pectin samples in 3 L scale. Values are means of 2 measurements \pm standard deviation. Values that do not share a letter within the column are significantly different ($p < 0.05$).

Enzyme/acid	Composition [%]						
	GalA	Ara	Fuc	Gal	Glu	Rha	Xyl
Laminex C2K	81.3 \pm 2.0 ^a	9.3 \pm 0.3 ^c	0.1 \pm 0.0 ^a	1.7 \pm 0.1 ^a	1.6 \pm 0.1 ^d	0.7 \pm 0.1 ^a	0.7 \pm 0.0 ^d
Validase TRL	83.1 \pm 0.7 ^a	8.2 \pm 0.2 ^d	0.1 \pm 0.1 ^a	4.1 \pm 0.1 ^c	0.3 \pm 0.0 ^{ab}	1.5 \pm 0.1 ^c	0.3 \pm 0.0 ^c
Multifect B	84.0 \pm 0.9 ^a	7.2 \pm 0.3 ^c	0.1 \pm 0.0 ^a	2.4 \pm 0.0 ^b	0.5 \pm 0.1 ^c	0.9 \pm 0.0 ^a	0.3 \pm 0.0 ^c
GC880	83.8 \pm 0.7 ^a	8.5 \pm 0.4 ^{de}	0.1 \pm 0.0 ^a	2.6 \pm 0.2 ^b	0.1 \pm 0.0 ^a	0.9 \pm 0.1 ^a	0.2 \pm 0.0 ^{bc}
HNO ₃ (A)	90.1 \pm 2.0 ^b	3.3 \pm 0.0 ^a	0.1 \pm 0.0 ^a	3.9 \pm 0.0 ^c	0.4 \pm 0.0 ^{bc}	1.4 \pm 0.0 ^c	0.2 \pm 0.0 ^{ab}
HNO ₃ (B)	90.4 \pm 1.7 ^b	5.0 \pm 0.0 ^b	0.1 \pm 0.0 ^a	2.7 \pm 0.0 ^b	0.2 \pm 0.0 ^{ab}	1.1 \pm 0.0 ^b	0.1 \pm 0.0 ^a

Table 5

Gelling, calcium sensitivity and enzymatic fingerprinting analyses of enzyme and acid extracted pectin samples in 3 L scale. Enzymatic fingerprinting express the amounts of galacturonic acid [%] released after two enzymatic treatments: with endo-polygalacturonase and endo- and exo-polygalacturonase. Pectin E81 and PGA taken from Limberg et al. (2000a, 2000b). Values are means of 2 measurements ± standard deviation. Values that do not share a letter within the column are significantly different (*p* < 0.05).

Enzyme	<i>M_w</i> ^a	°US-SAG	Calcium sensitivity	Enzymatic fingerprinting	
				Endo PG [%]	Endo + Exo PG [%]
Laminex C2K	69.2 ± 0.6 ^c	174.9 ± 0.1 ^b	0	0.11 ± 0.01 ^a	1.51 ± 0.01 ^b
Validase TRL	56.6 ± 0.4 ^a	168.6 ± 0.1 ^a	0.1	0.05 ± 0.02 ^a	1.41 ± 0.20 ^{ab}
Multifect B	91.2 ± 0.6 ^c	195.4 ± 0.3 ^d	0.1	0.04 ± 0.03 ^a	1.16 ± 0.04 ^a
GC880	82.3 ± 0.6 ^d	195.6 ± 0.2 ^d	0	<0.02	1.04 ± 0.03 ^a
HNO ₃ (A)	63.3 ± 0.5 ^b	182.2 ± 0.4 ^c	0.7	0.29 ± 0.28 ^{ab}	3.96 ± 0.01 ^d
HNO ₃ (B)	145.5 ± 5.5 ^f	226.4 ± 0.4 ^e	4.7	0.27 ± 0.01 ^b	2.60 ± 0.19 ^c
E81 ^b	90	nd	0.066	0.4	1.5
F31	90	nd	0.066	0.4	1.5
PGA ^b	39	na	0.066	39.9	57.4

nd: not determined.
na: not applicable.
^a Viscosity–average molecular weight.
^b Taken from Limberg et al. (2000a, 2000b).

anti-homogalacturonan monoclonal antibodies. The results are shown in a heatmap, where the colour intensity correlates to the antibody signal detected (Fig. 4). For all four enzymatically extracted pectin samples, antibody signals were only detected by monoclonal antibodies JIM7 (Clausen, Willats, & Knox, 2003) and LM20 (Verherbruggen, Marcus, Haeger, Ordaz-Ortiz, & Knox, 2009). Both antibodies are specific to partially methyl-esterified homogalacturonan having preference towards high methyl-esterified pectin. In the case of acidically extracted pectins, mostly relatively low signals from JIM7 and LM20 were detected compared to the enzymatically extracted pectin, suggesting lower degrees of esterification in the acid extracted samples. Furthermore, JIM5, LM18 and LM19 monoclonal antibodies exhibited binding to acid extracted samples. These three antibodies are specific for un-esterified and partially methyl-esterified homogalacturonan, binding preferably to low methyl-esterified pectins. These results stay in agreement with the measured degrees of esterification of the enzymatically and acidically extracted pectins, and further confirm the results of enzymatic fingerprinting. Standard pectin samples E81, F31 and F19 were included in the study as controls and all showed the expected antibody binding patterns.

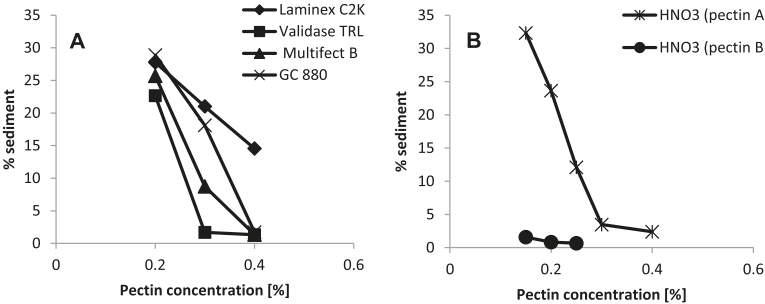


Fig. 3. Stabilization of milk drinks with enzyme (A) and acid (B) extracted pectins, expressed as the amount of sediment present in the milk drink vs. the amount of pectin added to stabilize milk drink. Standard deviation <1% for all measurement points.

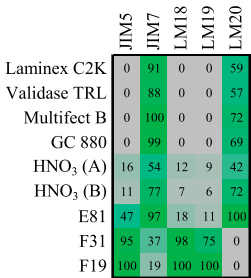


Fig. 4. Characterization of enzyme and acid extracted pectins using microarray technology. Extracted pectins were printed as microarrays and probed with 5 homogalacturonan specific monoclonal antibodies. The highest mean spot signal value obtained per each antibody was set to 100 and all other values were normalized accordingly. Results are presented in a heatmap, where colour intensity correlates to mean spot signal values. Antibody and sample names are listed at the top and at the left side of the heatmap respectively. Epitopes recognized by the probes are: partially methyl-esterified homogalacturonan (JIM7 and LM20) and un-esterified/partially methyl-esterified homogalacturonan (JIM5, LM18 and LM19). Standard lime pectin samples 81 (DE of 81%), pectin F31 (DE of 31%) and F19 (DE of 19%) were included as controls.

3.3. Enzymatic de-esterification of pectin for improved functionality

The pectin sample extracted with Laminex C2K was not able to stabilize casein molecules in the acidified milk drinks even at the 0.4% (w/w) addition level. Therefore it was enzymatically de-esterified to DE of approx. 70% to improve its functionality. Enzymes used for de-esterification were Rapidase FP Super, a pectin esterase derived from *A niger*, and Collupulin (papain), containing a high level of esterase activity. Calcium sensitivities of de-esterified pectins and their abilities to stabilize acidified milk drinks are presented in Table 6 and Fig. 5, respectively. Pectin treated with Rapidase FP Super was not sensitive to calcium, but stabilized acidified milk drinks at a 0.4% level, which was an improvement as compared with the 'mother' pectin extracted by Laminex C2K. Pectin treated with Collupulin had improved calcium sensitivity and stabilized the milk drinks already at the 0.2% level, which was a great functionality improvement as compared with Laminex C2K pectin. The reason for different functionalities of the two enzymatically de-esterified pectins was due to different origin of the two enzymes, used for removal of ester groups. Rapidase FP Super is a fungal enzyme, which is believed to produce homogeneous de-esterification of the pectin chain, whereas Collupulin, being of plant origin, results in a blockwise de-esterification pattern (Limberg et al., 2000b).

Table 6

Degrees of esterification and calcium sensitivities of enzymatically extracted pectins, de-esterified with Rapadase FP Super and Collupulin Liq.

Enzyme(s)	DE (%)	Calcium sensitivity
Laminex C2K	82.2 ± 2.5 ^b	0
Laminex C2K + Rapadase FP Super	70.3 ± 0.4 ^a	0
Laminex C2K + Collupulin Liq	69.7 ± 0.4 ^a	1.2

4. Discussion

Although GC 220 had the highest cellulase activity of all the enzymes used in the study, it resulted in the lowest pectin yields at all the pH regimes studied. This indicates that certain cellulase activities are probably more essential for pectin release than others. High level of endo-1,4- β -glucanase would probably contribute significantly to the amount of extracted pectin, due to its ability to break long cellulose chains into shorter fragments. Even though pectinolytic side activities detected in all enzymes were low, they were of high importance to the M_w of the released pectin polymer. It has been reported before that pectin released with enzymes suffer by having lower M_w than that of the acid-extracted pectin (Thibault, & De Dreu, 1988) and this fact could be attributed to the presence of pectinolytic activity, which was detected in all enzymes used in this study. Removal of this activity from the enzymes or addition of polygalacturonase, pectin lyase or RGI lyase inhibitors could preserve the molecular weight of pectin and improve its functional properties. However, Panouillé, Thibault, and Bonnin (2006) suggested that cellulase activity alone is insufficient to extract pectin from biomass with high yields. Partial hydrolysis of pectin by pectinolytic side-activities improves the accessibility of cellulose to cellulases and thus pectin release is enhanced. Therefore, it would be advisable to use purified enzymes for pectin extraction in order to assess the impact of the pectinolytic activities on the yield, M_w and functionality of the extracted polymer.

Pectin yields obtained in this study were much higher than those described in the literature for enzymatic extraction of pectin from citrus peel. Zykwincka et al. (2008) was able to obtain a yield of 12.6% (w/w) with Promod 24L and Cellulyve TR400 from citrus peel, whereas Lim, Yoo, Ko, and Lee (2012) extracted 7.3% (w/w) of low-galacturonan pectin from Yuza pomace with inferior functional properties, as compared to acid-extracted pectin. Although high pectin yield (22.2%) was recently obtained by Yu and Sun (2013), the enzymatic extraction had to be combined with microwave treatment of the peels, which significantly increased the pectin release. In contrast to the previous work, the present study demonstrated that enzymatic treatment solely can release pectin

with structural and functional behaviour similar to the classically acid extracted pectin.

Pectin samples released with enzymes had a very high DE (up to 82%), which was also observed by carbohydrate microarray analysis. High JIM7 and LM20 antibody signals and no JIM5, LM18 and LM19 detected binding in enzyme extracted pectins, and this suggested that mainly high DE pectins were present in these samples. Such a high DE is naturally observed in the lime fruit in the so-called “protopectin” and was preserved by the mild enzymatic process in the present study. Acid extraction removes ester groups, and DE typically falls to 70% or less. High degree of esterification in the enzymatic extracted pectin may be directly exploited commercially as the so-called Ultra-Rapid-Set pectin, which has a fast gelling at higher temperatures. Another ‘preserved’ feature is the level of neutral sugars. In acid extraction A and B, neutral hairy regions were degraded to a higher extent and their content did not exceed 9% in the final product. In commercial pectin products the neutral sugar contents are typically between 5 and 10% by weight (Nielsen, 1996). In the case of the enzymatic process, neutral sugar content varied between 12 and 15% which indicated a more native form of the polymer. Pectin samples had similar amount and composition of neutral sugars as already described (Zykwincka et al., 2008).

All four pectin samples extracted in the up-scaled processes were able to gel in the presence of 65% sugar. Gelling of enzymatically extracted citrus pectin was described before (Ptichkina, Markina, & Romyantseva, 2007; Zykwincka, Gaillard, Boiffard, Thibault, & Bonin, 2009). Although pectin extracted by Validase TRL had the lowest °US-SAG, it might be suitable for this application from the economy point of view, since it was extracted in much higher yields than pectin obtained by Multifect B or GC880, having much higher °US-SAG.

Pectin samples extracted with Validase TRL, GC 880 and Multifect B were able to stabilize milk drinks at the concentrations tested. Although their dose was higher than in the case of acid extracted pectin B, it was surprising that they were able to work in this application, taking into account their very high degrees of esterification, low amount of galacturonic acid in blocks, low calcium sensitivity and low viscosity—average molecular weights. Although it has been generally believed that high ester, calcium sensitive pectin is better for casein stabilization than non-sensitive pectin, too high of calcium sensitivity may cause an increase in the viscosity of the milk drink or even gelling due to presence of calcium in milk (Gerrish, 1998). Non-calcium sensitive acid extracted pectins providing good milk stability were previously described, suggesting there are other factors controlling milk stability than calcium sensitivity of the pectin used (Laurent & Boulenguer, 2002). Commercial pectin samples used for the stabilization of acidified milk drinks typically contain both calcium sensitive and non-sensitive pectin fractions (Gerrish, 1998). Pectin extracted with Validase TRL required lowest dosage for protein stabilization and could be regarded as a promising candidate for this application, taking into account very high yields of enzymatic extraction and lower energy consumption of the process. Moreover, enzymatic de-esterification of pectin extracted with Laminex C2K produced pectin with increased calcium sensitivity and ability to stabilize acidified milk drinks already at a concentration of 0.2%. The enzymatic process could be optimized further in terms of enzyme dose or extraction time, as shorter reaction time reduces energy input and positively impacts the economy of the enzymatic process.

Enzymatic extraction of pectin has been of interest for the food industry for many years but the drawbacks of the current extraction technology has been the efficiency of the enzymes and the selectivity, in addition to pectinolytic activity (Ptichkina et al., 2007). In this study we managed to extract pectin at a very high yield and the

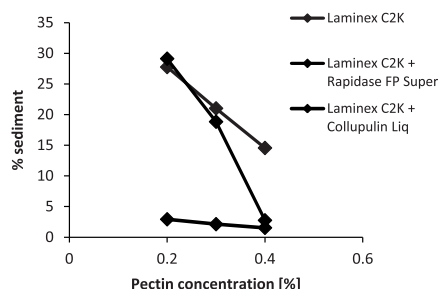


Fig. 5. Stabilization of milk drinks with pectin extracted with Laminex C2K and de-esterified by Rapadase FP Super and Collupulin Liq. Standard deviation <1% for all measurement points.

pectin moreover had functional properties equal or better than those of classically extracted pectin. By changing the pH of the process one could manipulate the yield and the length of pectin polymer. The highest yields were obtained at the highest pH used (4.8), but the high yields were accompanied by loss of pectin molecular weight. Pectin samples possessed functional properties, including gelling and stabilization of the milk drinks. Overall, the results prove that commercial enzymes are useful for extraction of the pectin from lime peel and present an alternative to the current technology, which eliminates the need for disposal of acidic wastewater. Further improvements of the enzymatic extraction process could be carried out by partial purification of the commercial enzymes to eliminate the pectolytic activities, similarly to the study of Byg et al. (2012), involving purification of commercial enzymes for extraction of RGI from potato pulp. Another fairly simple solution could be via genetic engineering to design *Penicillium funiculosum* or *Trichoderma longibrachiatum* strains, which produce Laminex C2K and Validase TRL, respectively. Deletion of the pectolytic genes, combined with addition of accessory genes encoding for enzymes, such as xyloglucanase and extensin degrading activity could pave the way for a novel large-scale enzyme based process for selective biomass opening, allowing value-added products to be recovered prior to utilization of the residual biomass in the bio-ethanol process. With the new genetically engineered *Penicillium* and *Trichoderma* strains it is possible to produce bulk amounts of the optimized pectin extraction enzymes and thereby lower the production cost to achieve an economically viable production. Alternatively, it is also possible to generate monocomponent enzymes produced in *Pichia pastoris* to accommodate a future study on enzymatic extraction with pure enzymes without galacturonan chain breaking activities, and to extract pectin with higher M_w and improved functional properties.

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3. Application of FTIR, carbohydrate microarrays and multivariate data analysis for prediction and characterization of enzyme- versus acid-extracted pectin.

3.1 Hypotheses and objectives

Pectin production is complex and final product quality assessment is accomplished at the end of the procedure, employing time-consuming off-line laboratory tests. Therefore, it would be of interest to pectin producers to shift the quality control upstream in the process and, in this way, enable continuous process monitoring and reduce the risk of process failures. The hypothesis of this study was that FTIR and carbohydrate microarrays are useful tools to predict pectin yield during the pectin production process by analyzing crude pectin extracts. To our knowledge, neither FTIR nor carbohydrate microarrays have been used before to predict the outcome of pectin extraction.

3.2 Key points

Time-resolved enzymatic and acidic pectin extractions have been conducted. In each case, samples of crude pectin extract were taken at specific time points and individual pectin yields were determined by propan-2-ol precipitation. First aliquot of each crude extract was used to acquire the mid-infrared spectrum between 950 cm^{-1} and 1550 cm^{-1} according to the method described in Andersen, S. K., Hansen, & Andersen, H. V. (2002). Second aliquot was analyzed by a microarray experiment, where pectin samples were deposited onto nitrocellulose membrane and probed with a panel of antibodies and carbohydrate binding modules (CBMs). Partial least squares regression (PLS) models were developed for enzymatic extraction and enzymatic plus acidic extractions, using the data obtained by FTIR and carbohydrate microarray. Additionally, principal component analysis (PCA) was utilized to analyze the spectral changes of enzymatic and acidic extractions.

3.3 Experimental considerations

Enzyme-extracted pectins possess higher amounts of neutral sugars than acid-extracted pectins, as substantiated in *Paper I*. In this study this fact was confirmed by the analysis of FTIR peaks of enzyme- and acid-extracted pectins. Microarray analysis, however, did not confirm the presence of neutral sugar side chains in case of enzyme-extracted pectin, which suggests that they were detached from the pectin chain and hence were not immobilized on glass slides. Smaller oligosaccharides and monosaccharides were not expected to immobilize onto the membrane and would therefore be washed off. If RGI side chains were degraded by the enzyme mixture, they would be cleaved into smaller fragments. These fragments were detectable by FTIR measurements of the extract solutions, but when printing extracts

containing these fragments, they would most likely be too small to immobilize efficiently and therefore they would not give rise to any antibody signals.

Additionally, FTIR and carbohydrate microarrays suggested that enzymatically extracted samples contained pectin with higher degree of esterification in relation to pectin extracted by HNO_3 , which was in agreement with the conclusions from *Paper I*. Moreover, the carbohydrate microarray results suggested that acid-extracted pectin contained additional regions with low degree of esterification, which could not be found in the enzyme-extracted pectin.

In carbohydrate microarray analysis, the specific binding of antibodies with specific epitopes is utilized to obtain information about the sample deposited on the array. Within a certain dynamic range, the higher the concentration of the deposited sample, the greater is the spot signal produced by antibody or CBM binding. Such arrays therefore provide direct information about the relative abundance of specific epitopes across the sample set tested. Nevertheless, the exact structure of the epitopes and the affinity of an antibody towards different epitopes are not known. Hence, the lack of antibody signal specific to e.g. HE pectin, does not mean that this structure is not present in the sample, but that some characteristic structural motifs (epitopes) are not present.

3.4 Conclusions

The study illustrated that it was possible to use FTIR and carbohydrate microarrays combined with chemometrics to predict pectin yield and characterize pectin crude extract. Both methods showed characterization abilities and helped understand the structural differences between acid- and enzyme-extracted pectin.

The results suggested a major difference between enzymatic and acidic crude pectin extracts in the degree of esterification and abundance of RGI pectic regions. While FTIR indicated high amounts of neutral sugars for enzymatic extracted samples, carbohydrate microarray results suggested that those oligosaccharides have been cleaved from the pectin and only existed independently in solution. That indicated the high relevance of choosing the appropriate enzymatic composition to obtain pectin with desired structural features.

FTIR, carbohydrate microarray and multivariate analysis for assessing pectin extracts.

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Abstract

Pectin production is complex and final product quality assessment is generally accomplished at the end of the process using time-consuming off-line laboratory analysis. In this study Fourier transform infrared spectroscopy (FTIR) and carbohydrate microarrays, combined with chemometrics, were employed to predict pectin yields and structural features. Using crude lime peel extracts, both FTIR and carbohydrate microarray analysis showed predictive and descriptive abilities on pectin samples extracted either by acid or enzymes. Furthermore, FTIR determined the optimal extraction time for the enzymatic and acidic extraction processes, respectively. The combined results of the two methods suggested major differences in crude pectin extracts generated either by enzymes or acid. Enzymatic extracted pectin showed both higher esterification (DE=82%) and had a more homogenous degree of esterification, whereas acidic extracted pectin had a lower degree of esterification (DE=67%) and showed very heterogeneous esterified pectin, when probed with the monoclonal antibodies JIM5, JIM7 and LM20.

Keywords: Pectin extraction, Chemometrics, Fourier transform infrared spectroscopy, Partial Least Squares Regression, Antibodies, Homogeneous esterification

Introduction

Pectin designates a family of plant cell wall polysaccharides, which are principally made up of four structural units: homogalacturonan, xylogalacturonan, rhamnogalacturonan type I (RGI), and rhamnogalacturonan type II (RGII). Homogalacturonan consists of an unbranched chain of α -1, 4-linked galacturonic acid residues, which may be methyl-esterified and/or acetylated, and which may be intervened by rhamnose residues (Voragen, Beldman, & Schols, 2001). Homogalacturonan is the quantitatively most dominant component in commercial “pectin”, which are the hydrocolloids used in foods and in other products such as pharmaceuticals, personal care, cosmetics, thickening and gelation agents. Pectin is approved for food use in the European Union (E440 (i) and E440 (ii)), and is usually produced by extraction from apple pomace and citrus peels (lemon, lime and orange). Recently, significant efforts have been made to develop pectin extraction methods that are more sustainable than the classical acid-assisted extraction, by e.g. using enzymes instead of acid (Zykwinska et al. 2008; Lim, Yoo, Ko, & Lee, 2012) or by employing new heating strategies in order to lower the energy expenditure (Rudolph, & Petersen, 2011). Nevertheless, current industrial practice for large scale pectin production involves extended acid extraction of the raw material, usually involving HNO_3 treatment for 3-12 h at 50-90°C (Rolin, Nielsen, & Glahn, 1998). Final pectin product quality assessment includes a number of classical, empirical laboratory tests such as measurements of intrinsic viscosity, calcium sensitivity, as well as size exclusion chromatography for molecular weight assessment and high performance liquid chromatography for monosaccharide composition analysis.

Fourier transform infrared spectroscopy (FTIR) is based on the measurement of absorption in the mid-infrared frequency range. Since organic molecules have intra- and intermolecular bonds that absorb in this frequency range, each spectrum obtained will reflect the chemical (structural) composition of the sample being analyzed. Hence, FTIR, in combination with appropriate chemometric methodology required to extract patterns in the spectra obtained, is a rapid, non-destructive analytical method (Kuligowski, Cascant, Garrigues, & de la Guardia, 2012), that can be employed for a number of complex, analytical applications, including quality assessment once proper calibrations have been developed. FTIR has thus proven useful for detecting e.g. the authenticity and adulteration of milk (Nicolaou, & Goodacre, 2010) and virgin olive oil samples (Rohman, & Man, 2010), and has more recently shown applicability within more advanced applications such as “temporal evolution profiling” for fingerprinting of enzymatic modifications and extractions of carbohydrates (Baum et al., 2013).

Carbohydrate microarrays are chip-based tools to study interactions of biomolecules with carbohydrates, and thus represent a different kind of methodology for advanced carbohydrate analyses than FTIR (de

Paz & Seeberger, 2012). Thousands of binding events can be assessed in parallel on a single slide with very small amounts of samples, making this analysis very high throughput (Fangel et al., 2012). Carbohydrate microarrays are applied in medical, animal, and prokaryote research (de Paz & Seeberger, 2012; Fangel et al., 2012), but have also been found to be a valuable tool in pectin research, and HG-directed monoclonal antibodies, differing in relation to pattern and degree of esterification have been developed and applied for analysis of plant cell walls (Clausen, Willats, & Knox, 2003; Sørensen & Willats, 2011).

The hypothesis of this study was that FTIR and carbohydrate microarrays might be useful in assessing pectin yields and notably for detecting subtle differences in enzymatically versus chemically extracted pectin. The objective of this work was therefore to assess the applicability and the knowledge obtainable by FTIR and carbohydrate microarrays. Using crude pectin extracts, obtained by acidic and enzymatic treatment of lime peels, both techniques were compared with respect to quantitative and qualitative analytical abilities, i.e. yield assessment and structural characterization, such as degree of esterification and levels of hairy regions of the differently extracted pectin.

Materials and Methods

Three pectin extractions were performed and both crude pectin extracts and precipitated pectin were examined. Pectin extraction I and II were conducted enzymatically using Laminex C2K, while extraction III was conducted using HNO₃. Extraction II, used for verification of the chemometric model, was conducted as extraction I, but with fewer time points (as detailed in Table 1).

Aliquots of the crude extractions I-III were analyzed using FTIR spectroscopy and carbohydrate microarray analysis and resulted in two separate data sets for individual chemometric analysis. The corresponding pectin yields were determined by propan-2-ol precipitation. These pectin yields were used as reference values for calibration of multivariate regression models. Samples from extraction II were used for testing and validation of the established chemometric models. The individual techniques and (chemometric) methods are described in further detail below.

Materials and chemicals

Dry lime peel was obtained from DuPont's pectin plant in Mexico (Dupont Nutrition and Biosciences Mexicana S.R.L., Tecomán, Colima, Mexico). The pretreatment involved milling of the peel to pass a 35-mesh size screen (centrifugal mill Retsch ZM 200, Haan, Germany). Several standard polysaccharides were used as benchmarks in the final analytical evaluation, namely pectin E81 from lime peel, with a degree of esterification (DE) of 81%, pectin F31 (DE 31%) derived from E81 treated with *Aspergillus*

niger's pectin methyl esterase (both from DuPont Nutrition Biosciences, Brabrand, Denmark), prepared as described in Limberg et al. (2000), and RGI from soy bean, purchased from Megazyme (Bray, Ireland). The commercial enzyme preparation, Laminex C2K, derived from *Penicillium funiculosum* (obtained from DuPont Industrial Biosciences, Leiden, The Netherlands) contained cellulase, xylanase and arabinoxylanase activities and a low level of pectinase activity.

Enzymatic extraction

For each pectin sample, 1.35 g of milled lime peel was mixed with 40 ml of 50 mM citric acid buffer pH 3.5. The mixture was heated to 50°C before addition of 75 µl of the enzyme Laminex C2K, equivalent to 235 carboxymethylcellulose-dinitrosalicylic acid activity units. After addition, the sample was incubated in a water bath at 190 rpm for 0-240 minutes (exact incubation times for extractions I and II are presented in Table 1). In each case, the reaction was terminated by heating at 100°C for 5 min to inactivate the enzyme. The crude extract was separated from the residual biomass by centrifugation for 10 min at 3500 rpm and stored at -20°C until further analyses.

Acidic extraction

For each acidic extracted pectin sample, 1.35 g lime peel was mixed with 40 ml deionized water and the mixture was heated to 70°C. The pH was adjusted to 1.7 with 42% nitric acid and the mixture was then incubated in a water bath at 190 rpm (more details about the incubation times in Table 1). The crude extract was separated from the residual biomass by centrifugation for 10 min at 3500 rpm and stored at -20°C until further analyses.

Determination of pectin yield

Pectin crude extracts were precipitated by mixing with a double volume of propan-2-ol, followed by agitation for one hour. The precipitated pectin samples were separated by filtration through a cotton canvas and dried overnight in a 40°C ventilated oven. Dried pectin samples were weighted and ground to <0.5 mm in an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany). Pectin yields, expressed in grams per 1.35 gram of the substrate, are presented in Table 1.

FTIR

An aliquot of crude extract was used to acquire the mid-infrared spectrum between 950 cm⁻¹ and 1550 cm⁻¹. All spectra were obtained using a MilkoScan™ FT2 (FOSS ANALYTICAL, Hillerød, Denmark). The instrument consisted of an interferometer, which scanned the IR spectrum using a cuvette with a path length of 50 µm. Acquisition was carried out at 42 °C with an optical resolution of 14 cm⁻¹. All spectra have been measured against an aqueous blank (FOSS Analytical, Hillerød, Denmark).

Carbohydrate Microarray analysis

Extracted pectin microarray printing

Crude extracts were 10-fold diluted with printing buffer (55.2% glycerol, 44% water, 0.8% Triton X-100). Each dilution was printed in triplicate with six drops per spot. A piezoelectric microarray robot, ArrayjetSprint (Arrayjet, Roslin, UK), equipped with a 12-sample high capacity JetSpyder sample pick-up device was used to deposit the crude extracts onto a nitrocellulose membrane with a pore size of 0.45 μm (Whatman, Maidstone, UK) at 22.5°C and 50% humidity. As positive controls, the three pectin samples, E81, F31 and RGI (described earlier) were also deposited on the nitrocellulose membrane.

Extracted pectin microarray probing

Microarrays were blocked for 1 hour in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.7 mM KH_2PO_4 , pH 7.5) containing 5% w/v low fat milk powder (5%M-PBS). Thereafter, microarrays were probed for 2 hours with a panel of 30 anti-rat antibodies, anti-mouse antibodies and carbohydrate binding modules (CBMs) diluted in 5% M-PBS to 1/10, 1/1000 and 10 $\mu\text{g/ml}$ respectively. The antibodies and CBMs were obtained from PlantProbes (Leeds, UK), INRA (Nantes, France), BioSupplies (Bundoora, Australia) and NZYTEch (Lisboa, Portugal), respectively. Afterwards arrays were washed with PBS and incubated for 2 hours in either anti-rat, anti-mouse or anti-His tag secondary antibodies conjugated to alkaline phosphatase (Sigma, Poole, UK) diluted 1/5000 (for anti-rat and anti-mouse) or 1/1500 (for anti-His tag) in 5 % M-PBS. After the incubation, arrays were washed with PBS and developed with a solution of 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl_2 , 100 mM diethanolamine, pH 9.5). Development was stopped by washing the arrays in deionized water.

Quantification of the data

Developed microarrays were scanned at 2400 dpi (CanoScan 8800F, Canon, Søborg, Denmark), converted to TIFF files and antibody and CBM signals were quantified using Array-Pro Analyzer 6.3 (Media Cybernetics, Rockville, USA). Results were presented in a heatmap, where the maximal mean spot signal was set to 100 and the rest of values normalized accordingly. A cut off of 5 was implemented and color intensity was correlated to mean spot signals.

Chemometrics

Chemometric modeling was done using MATLAB (The Mathworks Inc., MA, USA) and PLS Toolbox 6.0.1 (Eigenvector Research Inc., WA, USA).

Principal Component Analysis

Principal Component Analysis (PCA) is a descriptive chemometric method, which decomposes multivariate datasets into Principal Components (PCs) using loadings and scores (Hotelling, 1933; Wold, Esbensen, & Geladi, 1987). Principal Components represent orthogonal projections in the original multi-dimensional variable space which ideally characterize the main features of a data set. Normally, a low number of Principal Components is sufficient to express the measured variance of a (chemical) system, e.g. the IR spectral changes recorded during a time-resolved chemical extraction.

Partial Least Squares (PLS) regression

In contrast to PCA, Partial Least Squares regression is a supervised decomposition method, wherein a Y-block, typically containing reference analysis data, is used to find projections in the original multi-dimensional variable space, which are suitable for prediction of desired parameters (Beebe & Kowalski, 1987). Illustratively spoken, it rotates Principal Components, e.g. retrieved from PCA, in space to find maximal covariance between the X-block (multivariate dataset) and the Y-block (reference values), while minimizing the residuals in a least-squares sense. The resulting projections lead to latent variables, which allow prediction of one or more parameters from a newly measured sample. PLS calibrations can be employed to reveal data patterns from complex spectra (e.g. FTIR).

Validation

To obtain robust models using PCA/PLS, a number of decision criteria is required on how many principal components/latent variables are necessary to describe/predict the demanded quality measured during the (bio)-chemical process (Geladi, MacDougall, & Martens, 1985).

In this paper leave-one-out cross validation was used to choose the appropriate amount of latent variables for PLS. The number of PCs used for PCA has been chosen by monitoring the variance using different numbers of PCs.

Additional validation of the PLS models was achieved by calculating the root mean square error of prediction (RMSEP) for a test set (extraction II samples). This test set was independent from the established calibration models and suggested a suitable measure of model performance. The RMSEP was calculated using equation 1, where y_i and \hat{y}_i denote measured and predicted pectin yield, respectively.

$$RMSEP = \sqrt{\frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{n}} \quad \text{eq. 1}$$

Pre-processing

Chemometric pre-processing of data was necessary to remove irrelevant systematic variation in the data to gain better model prediction performance and to decrease the required number of factors necessary for

the analyses. The FTIR data were normalized using Standard Normal Variate (SNV) (Barnes, Dhanoa, & Lister, 1989) followed by mean-centering (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006). The carbohydrate microarray data were auto-scaled (van den Berg et al., 2006) and mean-centered. While SNV is typically used for spectroscopic techniques to remove light scattering effects, auto-scaling is used when variables indicate different units.

Monomeric composition of precipitated pectin samples

After hydrolysis of pectin in 1 M sulphuric acid (6 h, 100°C), the neutral sugars were quantitatively determined by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). Before hydrolysis, each pectin sample was washed three times in 80% ethanol, followed by washing one time in 96% ethanol to remove sugars. After hydrolysis, each sample was diluted and filtered through a 0.45 µm filter. The content of D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose and D-xylose in pectin was determined. The separation and quantification was performed by HPAEC-PAD using an ICS-3000 system (Dionex Corp., Sunnyvale, CA).

The columns used were CarboPacTM PA1 (4mm×250mm) analytical column and a CarboPacTM PA1 guard column. Eluent A consisted of deionised water and eluent B of 150 mM NaOH. 20 min isocratic elution with 15 mM NaOH was followed by 10 min washing with 150 mM NaOH. The flow was kept at 1 ml/min. Before injection of each sample (50 µl) the column was reequilibrated with 15 mM NaOH for 8 min. Analysis was made with the program Chromeleon 6.80 (Dionex Corp., Sunnyvale, CA). Galacturonic acid content was determined colorimetrically by the m-hydroxybiphenyl method in a microtiterplate format reaction (van den Hoogen et al., 1998) after hydrolysis for 1 h at 80°C in concentrated sulphuric acid with 0.0125 M tetraborate (Blumenkranz & Asboe-Hansen, 1973).

Results

Pectin was extracted by enzymes (extraction I and II) or by acid (extraction III), respectively (as detailed in Table 1), and aliquots of each of the crude extracts were sampled during the extraction period at specific time points. The crude pectin samples were measured by FTIR and carbohydrate microarray analyses for structural characterization and purity assessment of the pectin and for establishing pectin yield calibration models. Pectin yields were also determined for each sample by precipitation with propan-2-ol.

Table 1. Pectin yields (g), obtained by propan-2-ol precipitation of the crude pectin extracts released from 1.35 g starting material at a specific time point during enzymatic (extractions I and II) and acidic (extraction III) process. Values are means of 2 measurements.

Extraction time (min)	Pectin yield (g)		
	Extraction I	Extraction II	Extraction III
0	0.09	0.09	0.11
2.5	0.13		
5	0.14	0.12	0.19
7.5	0.15		
10	0.15	0.15	0.20
12.5	0.16		
15	0.17		
17.5	0.18		
20	0.18		
35	0.20		
30	0.21	0.22	0.26
35	0.23		
40	0.23		
45	0.23		
50	0.23		
55	0.23		
60	0.25	0.25	0.29
70	0.26		
80	0.27		
90	0.28		
100	0.29		
110	0.28		
120	0.29	0.30	0.31
240		0.31	0.33

FTIR

The time-resolved spectra of each of the three crude pectin extractions are shown in Figure 1. They are all difference spectra, which mean that the initial spectrum was subtracted from the whole series to eliminate background signals. The background signals contained various bands originating from the sample matrix (including enzymes, HNO_3 and salts), being present to a different extent in the enzyme- and acid-extracted samples. The first spectrum resulted in a zero line, while spectra at later stages evolved according to the release of soluble biopolymers (pectin) and oligosaccharides during extraction. The elapsing time is illustrated by grey scale, shifting from dark to light.

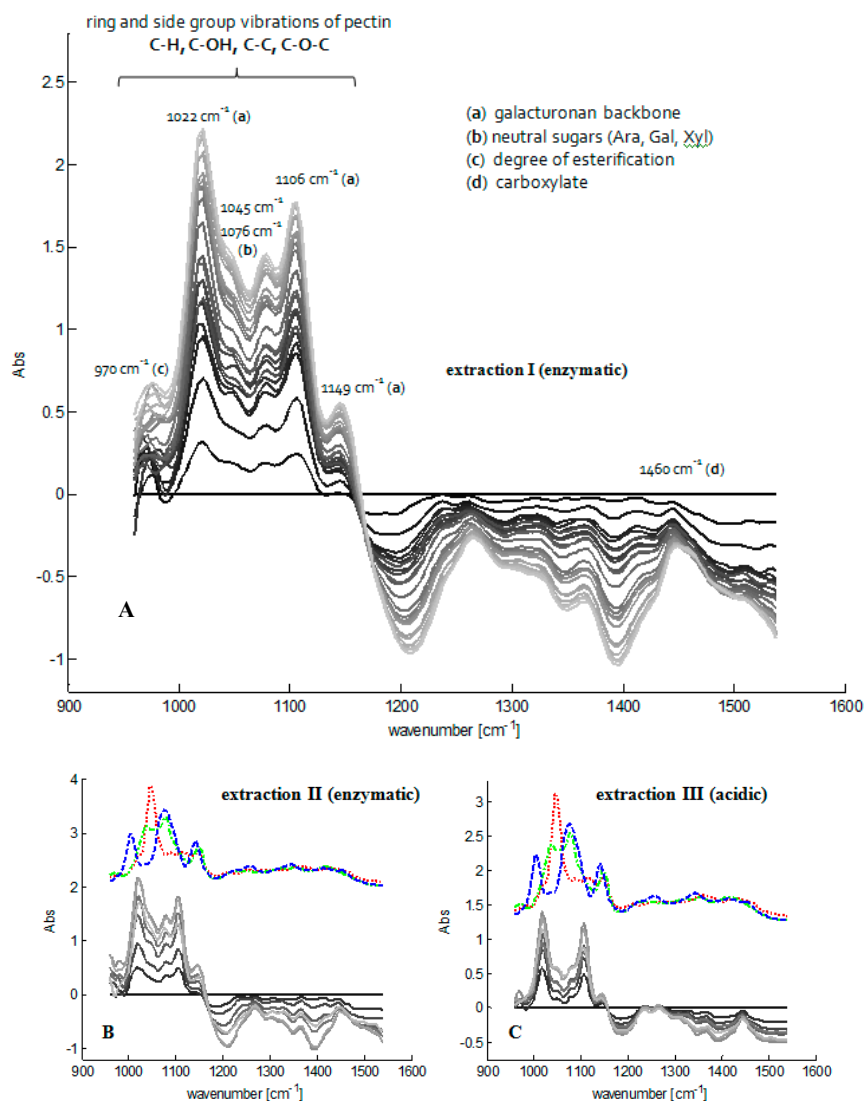


Figure 1. FTIR spectra of crude pectin samples, A) enzymatic extraction I, B) enzymatic extraction II and C) acidic extraction III. Changing grayscale from dark to light indicates elapsing extraction time. Spectra are displayed as difference spectra to highlight changes during extraction and to eliminate background signals. Spectral bands are identified in more detail in Figure 1A. While the homogalacturonan (a, 1022, 1106 and 1149 cm⁻¹) background signals are strong for both the enzymatic and the acidic extraction, the major difference can be found in the higher presence of neutral sugar signals (b, 1045 and 1076 cm⁻¹) for the enzymatic extraction. Degree of esterification, c, is given at 970 cm⁻¹. The free galacturonic acid, d, is given at 1460 cm⁻¹. Monosaccharide standard spectra are included in B and C for comparison (red-xylose, blue-arabinose, green-galactose).

When investigating the spectra of the three extractions, it appears that the spectral evolutions of extraction I and II are similar as expected, since these two extractions were done by enzyme catalysis, while the spectra from acidic extraction III evolve differently. Spectral changes resulting from differential signals due to interactions between extracted pectin and different solvents media are expected to be small (Wellner, Kačuráková, Malovíková, Wilson, & Belton, 1998). Hence, the differences in the evolution of the spectra of the acid- vs. the enzyme-extracted crude pectin samples are more likely a result of different molecular properties of the pectin in the two types of extracted samples.

Individual spectral bands for pectin, including the backbone and side group vibration bands, were identified in Figure 1A. The strong evolving peaks at 1022 cm^{-1} , 1106 cm^{-1} and less abundant at 1149 cm^{-1} (a) were due to the high homogalacturonan content (specifically due to C-C, C-O, CCH, OCH vibrations) as reported earlier (Coimbra, Barros, Rutledge, & Delgadillo, 1999; Fella, Anjukandi, Waterland, & Williams, 2009; Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000; Wellner et al., 1998). The band at 1149 cm^{-1} is characteristic for C-O-C vibrations of glycosidic linkages (+ glycosidic ring) (Wellner et al., 1998) and is therefore also characteristic for pectin backbone structures. Bands at 1045 cm^{-1} and 1076 cm^{-1} (b) were interpreted to be mainly caused by neutral sugars as arabinose, xylose and galactose (Coimbra, Barros, A., Barros, M., Rutledge, & Delgadillo, 1998). For comparison, the standard spectra of the three above mentioned sugars are included in Figures 1B and C. FTIR spectra detected higher amounts of neutral sugars in the enzymatic vs. the acidic crude extracts. This was especially evident when looking at the ratio of the two band groups (a) vs. (b) as shown in Figure 1. The intensities of the (b) bands were relatively high for enzymatic samples, which suggested higher amounts of neutral sugars in the enzymatic extracts (Figure 1 A and B), as compared to acidic extracts (Figure 1C). As neutral sugars are mainly found in side-chains of pectin rhamnogalacturonan I (RGI) structural domains, these bands could be interpreted as an indication of how branched the investigated pectin was or how pure the crude extract was. However, the bands might also be a response to the presumed free neutral sugars, which might be present independently in the crude extract.

An additional band at 970 cm^{-1} (c) indicated the degree of esterification. Accordingly, at 1460 cm^{-1} (d) one can observe a carboxylate band which tends to be weaker, when highly esterified pectin is present. Using the knowledge retrieved from Coimbra et al. (1998), Coimbra et al. (1999), Fella et al. (2009), Kačuráková et al. (2000), Wellner et al. (1998) and comparing the spectra with the monosaccharide standard spectra in Figure 1, it could be concluded that the degree of esterification (DE) of the enzymatic extracted pectin was higher than of acidic extracted pectin. However, the exact DE was not determined by

FTIR, as it was not the scope of this study. The DE was, however, quantified by chemical analysis, and the DEs for acidic and enzymatic extracted pectins were 67% and 82%, respectively (in agreement with the FTIR observation).

Principal Component Analysis

PCA was utilized to analyze the spectral changes of the crude pectin samples, extractions I and II in one common model (Figure 2A) and extraction III in a separate model (Figure 2B).

Both PCA models required only one Principal Component to describe 97.3% and 98.6% of the variance in the spectra, respectively. This was not surprising since the extraction pattern is expected to stay constant over the whole time period in both cases, meaning that the action of e.g. the enzyme does not change with time. The spectral loadings for both PCA models are given in Figure 2C and D and appear as expected for neutral sugar rich and less neutral sugar rich material, respectively.

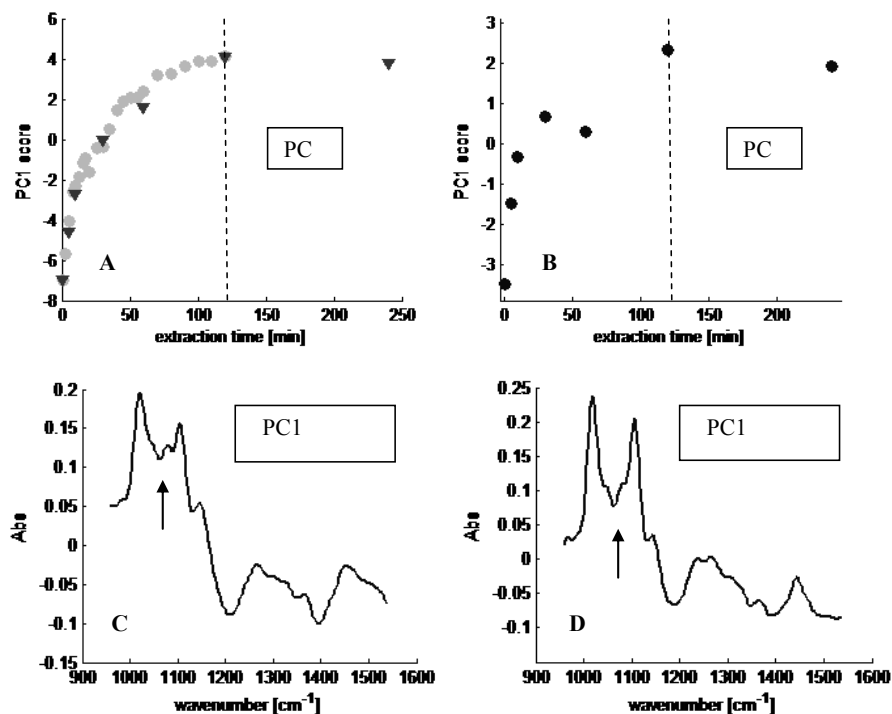


Figure 2. PC1 scores derived from PCA models plotted against extraction time for A) crude enzymatic extraction I (circles) and II (triangles) and for B) the crude acidic extraction III. As extraction time elapses, the spectral changes converge around 120 min in both cases, indicating that no additional pectin was released thereafter. PCA loadings are given in C) and D) and describe the main spectral features of enzymatic crude pectin extracts (extraction I and II) and acidic crude pectin extracts (extraction III). Characteristic band regions for neutral sugars are marked in both loadings with an arrow.

As shown in Figure 1, the spectra converge for all three extractions with elapsing time. In addition, the time-resolved scores of the individual PCA models also converge as shown in Figure 2A and B. This observation was confirmed by reference analysis of the pectin yield, prepared from the crude pectin samples by precipitation with propan-2-ol. In fact the Principal Component one scores of both PCA models correlated to a high extend ($R^2 > 0.92$) with the estimated pectin yield, determined by propan-2-ol precipitation. However, calibration models were approached using PLS and will be discussed in the following section.

Nevertheless, the evolution of three different crude pectin extractions could be monitored by FTIR. The method enabled possibilities to optimize the extraction time, as the spectral change after 120 min declined, and therefore indicated no release of additional pectin in all three extraction cases.

Partial Least Squares Regression

PLS models were developed for i) extraction I (model 1); and for ii) extractions I and III (model 2) as presented in Figure 3. In both cases the extraction II samples were used as a prediction test set for validation of the model. The calibrations are presented in Figure 3A and B, whereas their performance parameters are presented in Table 2. Calibration models were obtained using leave-one-out cross validation (calibration samples indicated as circles), while the pectin yields of the extraction II samples were predicted using the obtained models (triangles).

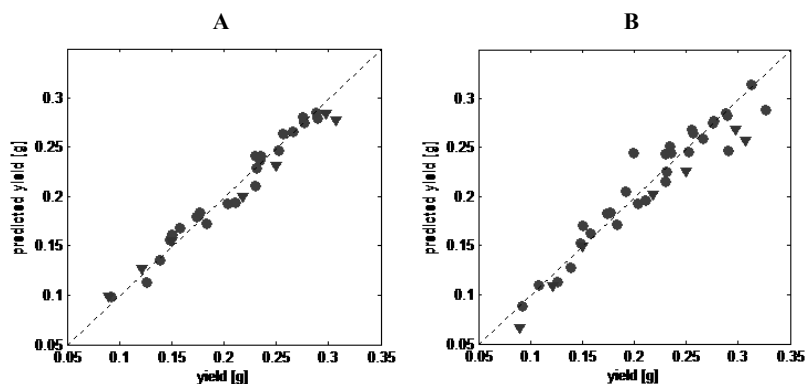


Figure 3. FTIR models A) PLS model 1 calibrated on crude enzymatic extraction I (circles) and tested on crude enzymatic extraction II (triangles). B) PLS model 2 calibrated on crude extraction I and III (acidic extraction) (circles) and tested on crude extraction II (triangles). Pectin yields (g), depicted on the x-axis, were obtained by propan-2-ol precipitation of the crude pectin extracts, released from 1.35 g of the starting material. Predicted yields (g), depicted on the y-axis, were obtained from PLS modeling of FTIR spectral data of the crude extracts. Yield of 0.3 g corresponds to $0.3/1.35=22\%$.

Table 2. Figures of merit for PLS models.

	FTIR		Microarray	
	model 1	model 2	model 3	model 4
R_{cal}	0.97	0.93	0.89	0.88
R_{pred}	0.99	0.98	0.91	0.73
RMSEP [g]	0.016	0.023	0.058	0.064

Leave-one-out cross validation indicated two latent variables for model 1 and four for model 2, respectively. In the previous section it was discussed that PCA models could be established using only one Principal Component to represent the spectral variances well. However, when looking at the spectra in Figure 1, one can observe small spectral shifts, especially for the bands around 1160 cm^{-1} and 1450 cm^{-1} . A second latent variable in model 1 compensated for this shifts and increased accuracy during calibration. In model 2 four latent variables were required, concerning i) the different extraction methods (acidic vs. enzymatic) resulting in two latent variables, ii) the spectral offset between the acidic and the enzymatic extraction, resulting from mean-centering (third latent variable) and iii) the spectral shifts as described for model 1 (fourth latent variable).

Both model 1 and model 2 could, with relative high confidence, predict the yield of the extracted pectin. Correlation coefficients for calibration were $R^2_{cal} = 0.97$ for the enzymatic extraction (model 1) and $R^2_{cal} = 0.93$ for the samples including acidic extraction (model 2), respectively. Prediction of the test set samples correlated with the reference values (correlation coefficients $R^2_{pred} = 0.99$ and $R^2_{pred} = 0.98$, respectively). All figures of merit, including the root mean square error of prediction RMSEP, are given in Table 2.

Carbohydrate microarray analysis

In carbohydrate microarray analysis, the specific binding of antibodies with well-defined sugar-binding epitopes is utilized to obtain information about the sample deposited on the array. The binding of the antibody is detected by the use of a fluorescently tagged or enzyme-linked secondary antibody. Within a certain dynamic range, the concentration of the deposited epitope co-varies with the spot signal, produced by antibody or CBM binding. Such arrays therefore provide direct information about the relative abundance of specific epitopes across the sample set being tested. The results of the carbohydrate microarray analysis of the three pectin extractions are shown in Figure 4 in a form of a heat-map. To evaluate the performance of the microarray, three pectin samples were included as positive controls. The samples were E81 (a), F31 (b) and RGI (c), and further information on their composition is given in Table 3.

From the heat-map data, assessing the microarray responses of 30 different antibodies and CBMs, it is evident that predominantly pectin related epitopes were detected in the crude pectin samples. There was, however, a clear difference in the profile of both the enzymatic extractions I and II and the acidic extraction III. All crude extracts obtained during the acidic extraction III showed interactions with mAb JIM5, a monoclonal antibody recognizing homogalacturonan with a low degree of esterification (DE). In addition, JIM5 also recognized the epitopes of the control pectin (b, F31) with high affinity, confirming the presence of a homogalacturonan structure with lower degree of esterification in the acidic extracted pectin. In contrast, no JIM5 signal was detected for any of the enzymatically extracted samples. Instead, relatively strong signals from mAb JIM7 and LM20, two monoclonal antibodies recognizing homogalacturonan with a higher DE, were observed suggesting that the enzymatic crude extracts contained pectin with a relatively high DE. It also indicates that the acid-extracted pectin contains both low and high DE pectin and therefore is much more heterogeneous in the methylation pattern than the enzyme-extracted pectin. The high degree of esterification (DE=82%) of enzymatic extracted pectin and the lower DE (67%) of the acidic extracted pectin (Table 3) corroborates with this observation in the micro-array.

PLS

When analyzing the obtained heat-map signals column-wise for each of the 30 antibodies/CBMs (Figure 4), none could be directly identified to correlate with the extracted amount of pectin. However, when combining all 30 antibodies and CBM responses using methods, such as PLS regression, a correlation did occur. Moreover, using PLS, the important descriptive and predictive antibodies were identified across the heatmap. As performed for FTIR, two models were established (Figure 5). Model 3 (Figure 5A) was calibrated on crude extraction samples I, while crude extraction samples II were used as the prediction test set. Model 4 (Figure 5B) was calibrated on crude extraction III, followed by validation using the crude extraction II samples.

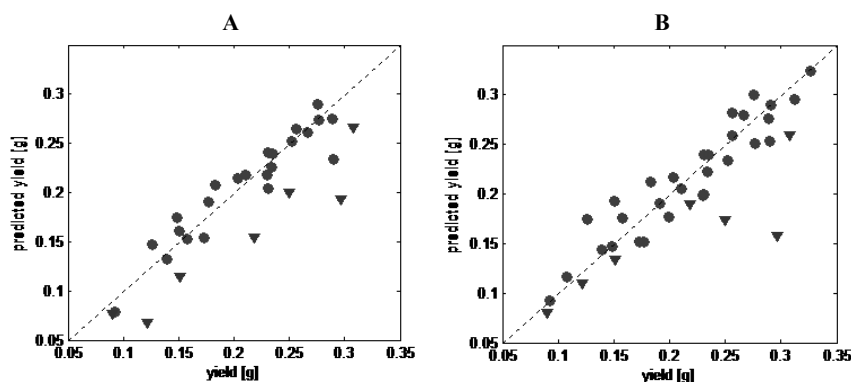


Figure 5. Microarray models A) PLS model 3 calibrated on crude extraction I (circles) and tested on crude extraction II (triangles). B) PLS model 4 calibrated on crude extraction III (circles) and tested on crude extraction II (triangles). Pectin yields (g), depicted on the x-axis, were obtained by propan-2-ol precipitation of the crude pectin extracts, released from 1.35 g of the starting material. Predicted yields (g), depicted on the y-axis, were obtained from PLS modeling of FTIR spectral data of the crude extracts. Yield of 0.3 g corresponds to $0.3/1.35=22\%$.

The established PLS models and their calibration parameters are presented in Figure 5 and Table 2. The correlation coefficients for the PLS calibrations were $R^2_{\text{cal}} = 0.89$ and $R^2_{\text{cal}} = 0.88$ for model 3 and model 4, respectively. Prediction test sets indicated correlations coefficients of $R^2_{\text{pred}} = 0.91$ and $R^2_{\text{pred}} = 0.73$ for model 3 and 4, respectively. These high R^2 values signified strong correlations in the data sets.

The number of latent variables was determined by leave-one-out cross validation. While only two latent variables were used for modeling the enzymatic extraction (model 3), five were necessary to build a combined model for acidic and enzymatic extraction (model 4). This was not surprising when looking at the microarray heat-map (Figure 4), showing that the signal pattern was very different for the enzymatic and the acidic extractions. Especially JIM5 antibodies and antibodies recognizing RGI regions gave strong response mainly for crude samples from the acidic extraction, which led to a higher rank in the data.

To reveal any covariance or associated properties in the different antibody binding patterns for enzymatic and acidic crude pectin extracts, a bi-plot of the first two latent variables from model 4 was created (Figure 6). Sample scores are indicated as triangles, while antibody loadings are illustrated as squares. While certain antibodies showed higher impact for PLS modeling, others seemed to be insignificant by showing zero abundance (or close to zero) on both loadings. This was expected due to the high degree of sparsity in the carbohydrate microarray heat-map. In other words, the signal was constantly zero for certain antibodies or did not significantly change during extraction. Hence, a zero loading abundance for those antibodies showed that they did not have predictive ability during enzymatic or acidic extraction.

On the other hand, highly predictive antibodies were JIM5, JIM7, LM20 and INRA-RU2, as indicated in Figure 6.

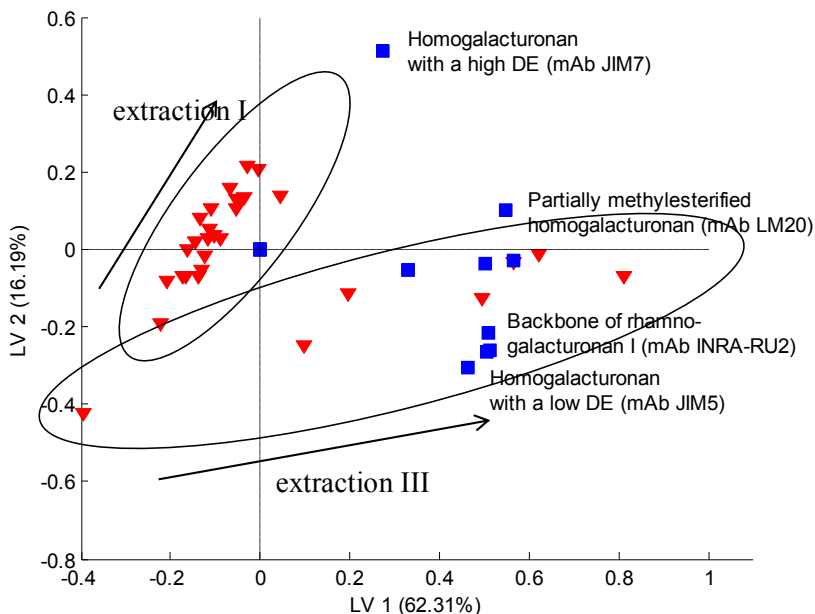


Figure 6. Biplot from microarray model 2 showing scores as triangles and monoclonal antibody loadings as squares. The first two latent variables describe the main differences between the acidic and the enzymatic crude pectin extracts. While crude enzymatic extracts contain homogalacturonan with a high DE, crude acidic extracts contain pectin with both higher and lower DE, as well as more RGI.

Scores and loadings with small in-between distances indicate associated properties, while a large distance between a score and a loading indicates the absence of a specific antibody binding. Enzymatic vs. acidic sample groups have been highlighted by ellipses. Since the first two latent variables distinguish mainly between extraction I (enzymatic) and extraction III (acidic), the main structural differences for enzymatic vs. acidic crude pectin extracts can be interpreted using this bi-plot. As the scores for enzymatic extracts move mainly towards the JIM7 loadings with increasing extraction time, pectin with high degree of esterification is expected to be extracted from the lime peel. However, scores for acidic extracts indicated extraction of structural domains with higher amount of RGI and lower degree of esterification. While the degree of esterification is expected to be lower for the acidic extracts, the relatively high amount of RGI backbone including neutral sugar side chains was not expected. The absence or much lower signals of antibody binding towards RGI, galactan and arabinan for the enzymatic extracted pectin can be explained

by the fact that the enzyme mixture might contain some arabinases, galactanases and RGI degrading enzymes, leading to decline of the corresponding antibody signals. All extracted liquids were spotted onto nitrocellulose membrane and polysaccharides bounded to the membrane by non-covalent passive adsorption. Smaller oligosaccharides and monosaccharides were not expected to be immobilized and would be washed off. If RGI side chains were degraded by the enzyme mixture, they would be cleaved into smaller fragments and most likely be too small to immobilize efficiently to the nitrocellulose membrane.

Chemical composition of precipitated pectin

To verify the information on the structure of acid- and enzyme-extracted pectin, provided by FTIR and carbohydrate microarray analyses, the chemical composition of the propan-2-ol precipitated pectin samples was determined. To compare the different responses of the antibodies and cell wall binding domains, the composition analysis of the three pectin standards were also determined. The content of galacturonic acid and neutral sugars was presented in Table 3.

Table 3. Chemical composition of enzymatic (extraction II) and acidic(extraction III) extracted pectin samples after 240 min reaction. Precipitation of both pectin samples was performed in propan-2-ol. Values are means of 2 measurements \pm standard deviation. Means that do not share a letter within a column are significantly ($p < 0.05$) different. Samples E81, F31 and RGI were added for comparison.

	Galacturonic acid	Arabinose	Fucose	Galactose	Glucose	Rhamnose	Xylose
Enzyme extracted pectin	81.3 \pm 2.0 ^{b***}	9.3 \pm 0.3 ^a	0.1 \pm 0.0 ^a	1.7 \pm 0.1 ^c	1.6 \pm 0.1 ^a	0.8 \pm 0.1 ^b	0.7 \pm 0.0 ^a
Acid extracted pectin	90.1 \pm 2.0 ^{a****}	3.3 \pm 0.0 ^b	0.1 \pm 0.0 ^a	3.9 \pm 0.0 ^a	0.4 \pm 0.0 ^b	1.4 \pm 0.0 ^a	0.2 \pm 0.0 ^b
F 31	74.5 [*]	0.2 \pm 0.0 ^c	0.1 \pm 0.0 ^a	3.3 \pm 0.2 ^b	0.1 \pm 0.0 ^d	1.0 \pm 0.1 ^b	0.1 \pm 0.0 ^c
E 81	78.7 [*]	0.2 \pm 0.0 ^c	0.1 \pm 0.0 ^a	4.0 \pm 0.1 ^a	0.2 \pm 0.0 ^c	1.3 \pm 0.0 ^a	0.1 \pm 0.0 ^c
RGI from soybean	51 ^{**}	3.4 ^{**}	10.3 ^{**}	12.3 ^{**}	n.d.	6.4 ^{**}	13.7 ^{**}

^{*}data from Limberg et al. (2000); ^{**}data from the manufacturer specification sheet; ^{***}Degree of Esterification DE = 82%; ^{****} Degree of Esterification DE = 67%

Enzyme-extracted pectin contained relatively higher levels of arabinose and galactose, glucose, rhamnose and xylose. On the contrary, acid- extracted pectin contained lower level of arabinose and significant amounts of galactose and rhamnose. The total neutral sugar level of enzyme-extracted pectin reached approx. 14%, whereas in case of acid-extracted pectin it was approx. 9% by weight. The higher content of neutral sugars in enzyme-extracted pectin was in agreement with the FTIR analysis on the crude pectin extract. On the contrary, in the crude extract, the carbohydrate microarrays detected higher amounts of RGI, galactan and arabinan epitopes in acidic extract as compared to the enzymatic process. In addition, the microarray also detected relatively more of homogalacturonan with a lower degree of esterification in the crude acidic pectin extract than in the enzymatic extract. As expected, the control sample, F31 (b), containing only 31% methyl esters, showed enhanced level of low ester pectin. The contemplation of the measurements thus showed that the microarray analysis detected relatively more heterogeneity in the crude acid extract than that of the enzymatic extracts. In contrast FTIR detected several neutral sugars in the crude enzymatic extract in comparison with that of the crude acidic extract. The data may reflect that some of the neutral pectin oligomers present in the enzymatic extract did not bind properly to the nitrocellulose membrane in the microarray, but further work is required to confirm this. Nevertheless, the analysis of the precipitated pectin composition (Table 3) showed that both extraction methods delivered a pectin product very rich in galacturonic acid – and since both methodologies gave a higher than 65% by weight of galacturonic acid in the precipitate, both methods were in essence valid pectin extraction methods.

Discussion

In this study two multivariate techniques, namely FTIR and carbohydrate microarray analysis were used to analyze crude pectin extracts before propan-2-ol precipitation. Both methods could predict the pectin extraction yield well using PLS modeling. PLS models 2 and 4, which included the crude acidic extraction III samples, had lower prediction performance for both FTIR and microarray analysis. In particular, the yield prediction ability for carbohydrate microarray data decreased more severely, when including the crude acidic extraction III. The higher RMSEP did not only result from a worse precision, but also from a bias induced by the prediction samples itself. This bias is evident by the fact that both models 3 and 4 predicted all test set samples (extraction II) with a lowering offset (Table 2). This bias was not recognizable for predictions using the FTIR models 1 and 2.

FTIR and carbohydrate microarrays provided the insight into the structural properties of the crude pectin extracts and provided complementary information. FTIR detected a high amount of neutral sugars in the enzymatic samples, which was in agreement with compositional analysis of precipitated pectins (Table 3)

and literature data (Nielsen, 1996; Zykwinska et al., 2008). On the contrary, carbohydrate microarray suggested lower amounts of RGI and neutral sugars in those extracts. In fact, it detected a low level of arabinan and neither RGI nor galactan in the analysed enzymatically extracted pectin sample (240 min extraction). However, the crude extracts investigated in this study contained probably mono- and oligomers, which would not be present in the pectin product after propan-2-ol precipitation. Additionally, short oligomers do not bind strongly to the nitrocellulose membrane and hence would not be detected by microarray analysis, but would be visible by FTIR. The results furthermore suggested that the enzymatic cocktail decreased the amount of neutral sugars in the extracted pectin over time, as the antibody responses of INRA-RU1 (RGI backbone) and LM5 (galactan) decreased with increasing extraction time. Further analysis would be necessary to determine, if the detected RGI and neutral sugars belonged to the pectin polymer or existed free in solution.

Additionally both FTIR and carbohydrate microarrays suggested that enzymatic extracts contained pectin with higher degree of esterification (DE=82%) in relation to pectin extracted by HNO_3 (DE=67%). Moreover, the carbohydrate microarray results suggest that acidic extracted pectin contained additional regions with low degree of esterification, which could not be detected in the enzymatic extracted pectin. This was not surprising as low pH enhances the hydrolysis of ester bonds and therefore reduces the degree of esterification in acidic extracted pectin (Rolin, Nielsen, & Glahn, 1998). Another explanation might be that a low level of polygalacturonase activity in the Laminex C2K preparation (unpublished data), would induce higher degradation of the non-esterified homogalacturonan stretches, thereby leaving high esterified homogalacturonan stretches more available for binding to the nitrocellulose and in turn respond in the microarray assay.

Conclusion

The present study illustrated that FTIR spectroscopy and carbohydrate microarray analysis have potential – in combination with multivariate data analysis - as analytical techniques for assessing crude pectin extracts and predict pectin yields and, to a certain extent, quality, notably with respect to degree of esterification of the pectin. Especially FTIR showed high accuracy and precision during PLS modeling and, being a rapid and non-invasive method, it therefore enables possibilities for further intelligent instrumentation and online monitoring. Furthermore, simple PCA on FTIR data indicated possibilities to determine the optimal extraction time without the need for interrupting the process or use cumbersome, classical reference analysis. The requirement for further exploitation of the FTIR in an on-line system is a simple calibration of the pectin yield (as shown in Figure 3) and a robot device for crude pectin sampling.

Both methods enabled comprehensive characterization abilities of the crude pectin extracts. The results suggested major differences between the enzyme- and the acid-extracted crude pectin extracts with respect to degree of esterification and abundance of RGI pectin regions. Although it was difficult to overview the complex results from the carbohydrate heat-map, multivariate tools as bi-plots derived from PLS helped to identify highly descriptive and predictive antibodies during calibration modeling. Further analysis would be necessary to determine if the detected RGI and neutral sugars were covalently linked to the pectin polymer or existed free in solution.

The high throughput microarray analysis might be used to quantify the final pectin yield and in turn be used to generate reference data for FTIR spectra and, in this way, replace time-consuming reference analysis, necessary for calibration modeling. Such calibration models could then be used for online monitoring. Hence, both FTIR and carbohydrate microarray analysis have high potential to move pectin analysis closer to pectin production, which is particularly important in industrial process and quality control.

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4. Prebiotic potential of sunflower pectin fractions

4.1 Hypotheses and objectives

Sunflowers are grown for their seeds, whereas the residues such as heads and stalks, are considered agricultural wastes (Lin, Sosulski, Humbert, & Downey, 1975). However, waste residues still contain approx. 7% protein, 3% lipids, and 70% carbohydrates, such as cellulose, hemicelluloses and pectin, which can be further extracted (Miyamoto, & Chang, 1992). The hypothesis of the study was that sunflower pectin has a prebiotic potential and that the pectin chain length influences the prebiotic effect. The purpose of the present study was, firstly, to efficiently extract pectins from sunflower heads and subsequently divide them into fractions according to size and, secondly, to evaluate the prebiotic potential of the fractions using *in vitro* fermentations.

4.2 Key points

Statistically designed, small scale extractions were performed to find optimum pH and temperature of the pectin extraction process. The selected conditions were applied in 3L scale, and the extracted pectin was divided into fractions using membrane filtration. The fractions were characterized and their prebiotic potentials were evaluated using *in vitro* fermentations.

4.2 Experimental considerations

Most pigments in sunflower heads are strongly associated with the pectin extract (Shi, Chang, Schwarz, Wiesenborn, & Shih, 1996). Pretreatment of sunflower pulp is therefore essential to remove those dark pigments and other contaminants, such as simple sugars, proteins and minerals. In this study, alcohol was chosen as the method for pulp washing, due to the highest visible removal of pigments and lowest apparent pectin loss. Additionally, alcohol washing has the advantage of being capable of partial inactivation of pectin methyl esterases, present in the sunflower plant (Sabir, Sosulski, & Campbell, 1976).

Water insoluble sunflower pectin can be extracted using of chelating agents, such as ammonium oxalate and oxalic acid (Lin et al., 1975; Sabir et al., 1976), polyphosphates e.g. sodium hexametaphosphate/ Calgon (Miyamoto, & Chang, 1992; Mohamadzadeh, Sadeghi-Mahoonak, Yaghbani, & Aalami, 2010; Sabir et al., 1976) or disodium ethylene diamine tetraacetic acid (EDTA) (Sosulski, Lin, & Humbert, 1978). In this study sunflower pectin was extracted with McIlvaine's buffer at different pH different temperatures in order to determine the conditions for the highest pectin yield.

The experiment was scaled up to 3L scale and the extracted pectin was fractionated by membrane filtration into the following three fractions: SPF>100 kDa, SPF50-100 kDa and SPF<50 kDa. The scale up of the extraction process from 40 ml to 3L resulted in lower pectin yields (approx. 26%), which could be attributed to more sample handling and the dialysis step, which removed remaining buffer salts and low Mw compounds. The average Mws of the SPF<50, SPF50-100 and SPF>100 fractions, measured by GPC (relative to standard dextrans), were 8 kDa, 16 kDa and 115 kDa, respectively. The measured Mws of the pectin fractions were lower than expected when compared to the cut-off values of the membranes that they were filtered through. There could be several reasons explaining this phenomenon. First of all, pectin molecules in solution have a tendency to aggregate and low ester pectins are more susceptible to aggregation than high ester pectins. Secondly, membrane systems are designed for filtration/concentration of protein solutions. Polysaccharides in solution have larger hydrodynamic volumes than proteins of the same Mws. Therefore, a membrane with a cut-off value of eg. 50 kDa may in fact allow passing polysaccharide molecules of much lower Mw.

4.4 Conclusions

It was possible to extract sunflower pectin in large amounts, to fractionate it and to test the biological activity of the size fractions. Fermentation of fecal slurries, obtained from 5 human subjects was used to evaluate the bioactivity of three sunflower pectin fractions. Results from *in vitro* fermentations showed the selective stimulation of *Bifidobacterium* spp. only in case of SPF>100. SPF<50 and SPF50-100 did not increase the density of Bifidobacteria compared to the negative control (NEG). Additionally, none of the three sunflower pectin fractions or FOS stimulated the selective growth of *Lactobacillus* spp.

None of the three sunflower pectin fractions caused significant changes in the densities of *Bacteroidetes* and *Firmicutes* after fecal fermentations. In the case of FOS, the relative ratio of *Bacteroidetes* decreased significantly ($P < 0.05$) compared to NEG, which could be due to an increase in other types of bacteria (Holck et al., 2011b). However, the increase of *Firmicutes* to *Bacteroidetes* ratio, observed in the case of fermentation with FOS, may potentially increase the obesity risk and influence the development of obesity-associated metabolic syndrome (Holck et al. 2011b; Ley, Turnbaugh, Klein, & Gordon, 2006; Turnbaugh et al., 2006). It was therefore beneficial that SPF>100, having a bifidogenic potential, did not affect the ratio between the two phyla.

Evaluation of the prebiotic potential of sunflower pectin fractions.

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Abstract

Sunflowers are grown for their seeds, which are rich in edible oil, whereas the residues such as heads and stalks are considered agricultural wastes. The content of pectin in sunflower heads varies between 15 and 24%, depending on the cultivar and the stage of harvest. The purpose of the study was to efficiently extract pectins from sunflower heads and to evaluate their prebiotic potential using *in vitro* fermentations. Sunflower pectin was extracted with McIlvaine's buffer at pH between 3 and 7 and at temperatures between 30 and 60°C, in order to determine the conditions of highest pectin release. The highest pectin yield was obtained at pH 7 but temperature was found to be insignificant. Sunflower pectin was fractionated into three fractions SPF<50, SPF50-100 and SPF>100 using 50 and 100 kDa membranes. The density of human fecal *Bifidobacterium* spp. was significantly higher ($p<0.05$) after fermentation with SPF>100 kDa compared to when no substrate was added. None of the three sunflower pectin fractions influenced the level of *Lactobacillus* spp., *Bacteroidetes* or *Firmicutes*.

Keywords: sunflower pulp, pectin, prebiotic, *Bifidobacterium*, *Lactobacillus*

Introduction

As people become more aware of the health implications of their diets, the market for prebiotic substances is growing rapidly. The development of novel prebiotics with better functionalities is possible by exploring new biomass sources, such as plant cell wall carbohydrates obtained from waste biomass, with the help of enzymes and advanced process knowledge. Recently, there has been much interest in the novel prebiotics obtained from cell wall polysaccharides, such as arabinoxylans or pectins. Pectin and pectic oligosaccharides, derived from different plant sources, have been found to promote the growth and activity of beneficial gut microflora and inhibit pathogen growth (Holck et al., 2011a; Holck et al., 2011b; Hotchkiss, Olano-Martin, Grace, Gibson, & Rastall, 2003; Thomassen, Vignæs, Licht, Mikkelsen, & Meyer, 2011). Bifidogenic fibers from potato pulp with molecular masses above 100 kDa increased the densities of *Bifidobacterium* spp. and *Lactobacillus* spp. 2–3 times more than fructo-oligosaccharides (FOS) (Thomassen et al., 2011). Moreover, by manipulation of the size of pectic oligosaccharides derived from sugar beet pectin, one could affect the ratio of *Bacteroidetes* to *Firmicutes*, which is believed to play a role in the development of obesity (Holck et al., 2011a). Pectic oligosaccharides have been found to be more bifidogenic than their parent pectins. Furthermore, pectins with lower degrees of esterification (DE) and pectic oligosaccharides have been found to be more prebiotic than high DE material (Hotchkiss et al., 2003).

Sunflowers are grown for their seeds, which are rich in edible oil, whereas the residues such as heads and stalks are considered agricultural wastes (Lin, Sosulski, Humbert, & Downey, 1975). However, the waste residues still contain approx. 7% protein, 3% lipids, and 70% carbohydrates, such as cellulose, hemicelluloses and pectin that can be further extracted (Miyamoto, & Chang, 1992). Pectin content in the sunflower heads varies between 15 and 24%, depending on the cultivar and stage of harvest (Miyamoto, & Chang, 1992). Sunflower pectin is strongly bound to divalent metal ions in the cell wall (Lin et al., 1975). The water insoluble pectin fraction can be extracted using chelating agents, such as ammonium oxalate and oxalic acid (Lin et al., 1975; Sabir, Sosulski, & Campbell, 1976), polyphosphates e.g. sodium hexametaphosphate/Calgon (Miyamoto, & Chang, 1992; Mohamadzadeh, Sadeghi-Mahoonak, Yaghbani, & Aalami, 2010; Sabir et al., 1976) or disodium ethylenediamine tetraacetic acid (EDTA) (Sosulski, Lin, & Humbert, 1978). Application of sunflower pectin in the food industry is currently very limited and restricted to utilization of its gelling ability (Iglesias, & Lozano, 2004; Kim, Sosulski, & Campbell, 1978).

The hypothesis of this study was that sunflower pectin, low ester type pectin, has a prebiotic potential and that the pectin chain length influences the prebiotic effect *in vitro*. The purpose of the present study was,

firstly, to efficiently extract pectins from sunflower heads and subsequently divide them into fractions according to size and, secondly, to evaluate the prebiotic potential of the fractions using *in vitro* fermentations.

Methods

Chemicals

D-galactose, L-arabinose, L-rhamnose monohydrate, D-fucose, polygalacturonic acid from orange and 3-hydroxybiphenyl were purchased from Sigma-Aldrich (Steinheim, Germany). Disodium tetraborate-10-hydrate, D-xylose and D-glucose were purchased from Merck (Darmstadt, Germany). Fructo-oligosaccharide (FOS) (2-8 DP) (Orafti®95) was purchased from Beneo-Orafti (Tienen, Belgium).

Sunflower head pulp preparation

Fresh sunflower heads (*Helianthus annuus L.*) were harvested directly from the field and transported to the laboratory. After seed removal, the sunflower heads were rinsed in deionized water to remove sand and other impurities. Each head was cut into four fragments and each fragment was ground using a kitchen blender and immediately placed in the microwave oven for blanching – to inactivate endogenous pectin methyl esterases (2 minutes at 1470 watt in Husquarna Micronett, QN 2276F, UK). The sunflower head pulp was placed in the microwave in approx. 100 g portions to ensure homogenous heat transfer. After blanching, the pulp was placed in a 50°C ventilated oven for 48 h for drying. The dried pulp was milled to 35 mesh in an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany).

Washing of the sunflower head pulp

Three methods of pulp washing were tried, namely (i) hot water washing (75°C) for 15 minutes at 1:25 solid: water ratio, according to the method by Mohamadzadeh et al. (2010), (ii) alkaline washing at pH 7.5 for 25 minutes at 1:28 solid: water ratio, according to the method by Shi, Chang, Schwarz, Wiesenborn, & Shih (1996) and (iii) alcohol washing (80% ethanol, 30 minutes, 90°C, 1:40 solid: water ratio) according to the method by Sabir et al. (1976). The results were compared on the basis of the colour of the pectin after washing and the colour and viscosity of the washing medium.

Small scale extractions of sunflower pectin for determination of optimum extraction conditions

A randomized full-factorial 3-levels design was used to determine the conditions for highest pectin yield. The design contained 9 combinations of the following two factors: pH 3-7 and temperature 30-60°C. The

center point (pH 5, 45°C) was measured in triplicate. The investigated response was pectin yield. A scheme of experimental design is presented in Table 1.

Table 1. Factors and their levels used in the experimental design.

Experiment	pH	Temperature[°C]
1	3	30
2	5	30
3	7	30
4	3	45
5	5	45
6	7	45
7	3	60
8	5	60
9	7	60
10	5	45
11	5	45
12	5	45

All pectin extractions were carried out for 1h in McIlvaine's buffer with 1:30 pulp-buffer ratio. 1.35 g of sunflower pulp was mixed with a pre-heated buffer and incubated in a shaking waterbath at 190 rpm. The released pectin polymer present in solution was separated from the residual biomass by centrifugation for 10 min at 3500 rpm (Varifuge 3.0, Heraeus Sepatech, Osterode, Germany). After centrifugation, the resulting solution was filtered through a Celite 545 (Celite France S.A., Nanterre Cedex, France) and the pH was adjusted to 4.0 with 5 M citric acid. Pectin was precipitated by mixing the pectin solution with an equal volume of propan-2-ol, followed by agitation for 1 hour. The precipitated pectin was washed in 50% propan-2-ol for 1 hour. It was separated by filtration through a cotton canvas and dried overnight in a 40°C ventilated oven. Dried pectin was ground to <0.5 mm in an Ultra Centrifugal Mill ZM 200 (Retsch, Haan, Germany).

3L upscale extraction of pectin

Up-scaling of pectin extraction to 3L volume was performed using a 3-neck round bottom flask with an agitator inside, which was placed in a heating mantle. The pulp: water ratio was the same as in the small scale extraction. The experiment was performed as described for the small scale process, except for the following modification: before precipitation, the pectin solution was filtered through a 10 kDa polyethersulfone spiral wound ultrafiltration module (Milipore) to remove buffer salts.

Determination of pectin yield

The yield of pectin was calculated as a weight ratio of the extracted pectin to sunflower pulp (both on dry matter basis).

Fractionation of the pectin using membrane filtration

A 0.5% pectin solution was prepared by dissolving pectin in hot (50°C) deionized water with vigorous mixing. Next, an overnight filtration through a 100 kDa cross-flow PES membrane (Sartorius, Goettingen, Germany) was performed. The retentate was defined as SPF>100 (Mw>100 kDa). The permeate was then further filtered through a 50 kDa cross-flow PES membrane (Sartorius, Goettingen, Germany) and defined as SPF50-100 (Mw between 50 kDa and 100 kDa). The permeate was defined as SPF<50 (Mw<50 kDa). Due to the high volume of the SPF<50 fraction (approx. 8L), it was concentrated using a rotary evaporator. All three pectin fractions were freeze-dried and stored at -20°C until further analysis.

Determination of galacturonic acid

Galacturonic acid (GalA) content was determined using the colorimetric m-hydroxybiphenyl method (Blumenkrantz, & Asboe-Hansen, 1973). The measurements were performed in a microtiter plate, according to the method by van den Hoogen et al. (1998), using a Tim-200 plate reader equipped with 492 nm filter (Teknunc A/S, Roskilde, Denmark).

Determination of the degree of methylation and acetylation of the pectin fractions.

The methanol and acetic acid contents of SPF<50, SPF50-100 and SPF>100 were determined according to the method by Melton and Smith (2001). Briefly, 30 mg of each pectin fraction was saponified with 1 ml of cold (4°C) isopropanol/NaOH solution for 2 hours at room temperature. Next, the suspensions were centrifuged at 2000×g for 10 minutes and the supernatants were analyzed by HPLC for methanol and acetic acid contents. The HPLC system used was a Summit LC (Dionex Corp., Sunnyvale, CA) equipped with a P680 HPLC pump, an ASI-100 automated sample injector, and an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad Labs, Richmond, CA, USA) in combination with a guard column (AG50W-X4. 50 × 4.6 mm, Bio-Rad Labs). 5mM H₂SO₄ was used as the eluent at a constant flow rate of 0.6 ml/min at 30°C. Components eluting from the column were detected with a Shodex RI-101 (Showa Denko KK, Kawasaki, Japan) refractive index detector thermostated at 40°C. Methanol and glacial acetic acid were used as standards.

After determination of galacturonic acid content of each pectin fraction, degrees of methylation and acethylation were calculated from the formulas:

Degree of methylation = (millimoles methanol/millimoles uronic acid) \times 100

Degree of acetylation = (millimoles acetic acid/millimoles uronic acid) \times 100

Neutral sugar composition

After hydrolysis of pectin in 1 M sulphuric acid, the neutral sugars are detected by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). Before hydrolysis, the pectin sample was washed three times in 80% ethanol followed by washing one time in 96% ethanol to remove sugars. The hydrolysis in 1 M sulphuric acid was carried out at 100°C for 6 hours. After hydrolysis the sample was diluted and filtered through a 0.45 μ m filter. The content of D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose and D-xylose in pectin was determined. The separation and quantification was performed by HPAEC-PAD using an ICS-3000 system (Dionex Corp., Sunnyvale, CA). The columns used were CarboPacTM PA1 (4mm \times 250mm) analytical column and a CarboPacTM PA1 guard column. Eluent A consisted of deionised water and eluent B of 150 mM NaOH. 20 min isocratic elution with 15 mM NaOH was followed by 10 min washing with 150 mM NaOH. The flow was kept at 1 ml/min. Before injection of each sample (50 μ l) the column was reequilibrated with 15 mM NaOH for 8 min. Analysis was made with the program Chromeleon 6.80 software (Dionex Corp., Sunnyvale, CA).

Determination of molecular weight (Mw) by gel permeation chromatography (GPC)

Mw of the pectin fractions was determined using gel permeation chromatography. Two 3.000Å and one 100Å (PSS, Mainz, Germany) GPC SUPREMA-LUX columns were used for gel permeation chromatography. Pectin solutions (1 g/L) were filtered through a 0.45 μ m filters before injection. The mobile phase was 0.05 M LiNO₃ with 0.02% NaN₃ which was filtered through a 0.22 μ m filter before use. Separations were carried out at 40°C with a flow rate of 0.6 ml/min. The eluent was monitored by a refractive index detector Optilab reX (Wyatt Technology Corporation). Molecular mass markers used were dextrans with molecular masses of 1080, 4440, 9890, 21400, 43500 and 66700 Da. The data were analyzed using Astra 5 software (Wyatt Technology Corporation).

Analysis of the biological activity of sunflower pectins

Subjects and fecal sample collection

Fecal samples were obtained from five healthy volunteers (three women and two men). None of the participants had been treated with antibiotics for at least 3 months before enrolment and had no history of gastrointestinal disorders. The fecal samples were collected in airtight containers at home by the participants and stored at 4°C (limited storage time was encouraged, in accordance with Ott et al., 2004),

until delivery to the laboratory, where they were processed immediately. The fecal samples were homogenized in 50 % glycerol (1:1 dilution) in an anaerobic cabinet (Macs Work Station, Don Whitley) containing 10% H₂, 10% CO₂, and 80% N₂, and stored at -80°C until further analysis.

In vitro fermentation by human fecal bacterial communities

Small scale fermentation studies were carried out to assess the effect of sunflower pectin fractions on the microbial composition in human fecal samples, in a similar manner to the method reported previously (Holck et al., 2011b). Parallel incubations with the established bifidogenic substrate, FOS, (Rossi et al., 2005) and no carbohydrates (NEG), respectively, which were used as controls. Fecal samples, prepared as described above, were defrosted in an anaerobic cabinet and 10% (w/v) fecal slurries were prepared by mixing the samples with anoxic phosphate buffered saline (PBS) (Oxoid, Greve, Denmark) immediately before fermentation. The carbohydrate sources were added to an autoclaved minimal basal medium (MBM) to give a final concentration of 5 g (dry matter)/l with reaction volumes of 2 ml. The MBM contained 2 g/l of peptone water, 1 g/l of yeast extract, 0.1 g/l of NaCl, 0.04 g/l of K₂HPO₄, 0.04 g/l of KH₂PO₄, 0.01 g/l of MgSO₄·7H₂O, 0.01 g/l of CaCl₂·2H₂O, 2 g/l of NaHCO₃, 0.5 g/l of bile salts, 0.5 g/l of l-cysteine hydrochloride, 0.005 g/l of hemin, 10 µl/l of vitamin K1 (0.02 mM), 2 ml/l of Tween 80, and 1 ml/l of 0.05% (w/v) resazurin solution. The pH of the final solution was adjusted to 7. Positive controls were made by adding FOS (5 g/l) to the MBM and negative controls were prepared by not adding any fibers to the MBM. All solutions were reduced overnight in an anaerobic cabinet and inoculated with fecal slurry, prepared as described above to a final concentration of 1% feces. Each fermentation was carried out in triplicate for each fecal community, carbohydrate source and controls. The fermentation assay was non-pH controlled and non-stirred due to the low reaction volume (2 ml) and was carried out in an anaerobic cabinet at 37°C. After 24 hours, each fermentation sample was centrifuged at 13000 rpm for 8 min. Pellets were used for extraction of bacterial DNA, as described below.

Extraction of bacterial DNA

DNA was extracted from the fermentation samples using the QIAamp Stool DNA Mini Kit (Qiagen, Hilden, Germany) with a bead-beater step in advance. The purified DNA was stored at -20°C until use.

Real-Time PCR assay conditions

Amplification and detection of purified bacterial DNA by real-time PCR were performed with the ABI-Prism 7900 HT from Applied Biosystems using optical grade 384-well plates. PCR reagents and DNA were loaded on the 384-well plates using the EpMotion pipetting Robot (Eppendorf). Primers specifically targeting 16S rRNA gene sequences of the total bacteria, the *Bacteroidetes* and *Firmicutes* phyla and the *Lactobacillus* and *Bifidobacterium* genera were included in the qPCR analysis (Table 2).

Table 2. 16S rRNA primers used for q PCR for quantification of the level of *Bifidobacterium* spp, *Lactobacillus* spp., *Firmicutes* and *Bacteroidetes* in the *in vitro* fermentations of the fecal samples with sunflower pectin fractions SPF<50, SPF50-100 and SPF >100.

Target gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)	Ref.
<i>Bifidobacterium</i> spp.	gcgtgcttaacacatgcaagtc	cacccgtttccaggagctatt	126	Penders et al., 2005
<i>Lactobacillus</i> spp.	agcagtagggaatcttcca	caccgctacacatggag	341	Heilig et al., 2002; Walter et al., 2000
<i>Firmicutes</i>	ggagyatgtggtttaattcgaagca	agctgacgacaaccatgcac	126	Guo et al., 2008
<i>Bacteroidetes</i>	ggarcatgtggtttaattcgatgat	agctgacgacaaccatgcag	126	Guo et al., 2008
Total bacteria	actcctacggaggagcagcagt	gtattaccgcggtgctggcac	200	Walter et al., 2000

Prior to quantification, all primers were tested to confirm sensitivity and specificity using DNA from pure bacterial species (Bergstrom et al., 2012). Each amplification reaction was done in duplicate for each of the triplicate fermentation samples in a final volume of 11 µl containing; 5.50 µl SYBR® Green Master Mix (Applied Biosystems, Denmark), 200 nM of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany), 2 µl template DNA (1 ng/µL), and Nuclease-free water purified for PCR (Qiagen). The amplification program comprised one cycle at 50°C for 2 min; one cycle at 95°C for 10 min; 40 cycles at 95°C for 15 sec.; 60°C for 1 min, and finally one cycle of melting curve analysis for amplicon specificity at 95°C for 15 sec, 60°C for 20 sec. and increasing ramp rate by 1.92°C/min until 95°C for 15 sec. The qPCR data was baseline corrected and N_0 -values, representing initial concentrations of the specified 16S rRNA genes, were calculated using the LinRegPCR software (version 11.1, based on Ruijter et al., 2009). All results were calculated as means of duplicate N_0 estimations. The relative quantities of gene targets encoding 16S rRNA sequences of the bacterial taxa were calculated using N_0 (bacterial target)/ N_0 (total bacterial population).

Results

Pulp washing

The sunflower pulp used in the study was washed in three ways, by (i) hot water, (ii) base and (iii) alcohol to remove pigments and impurities. In case of water and base washing, the colour of the pulp remained unchanged (dark brow/black) and the washing medium became viscous, suggesting pectin loss during washing. In case of alcohol washing, the color of pectin was significantly improved (light yellow) and the washing medium became very dark, which suggested that pigments were removed from the pectin. There

was no apparent viscosity increase of the washing medium after alcohol washing, which suggested lower pectin loss than in the case of hot water or base. Therefore alcohol washing was chosen as the method of pulp washing.

Determination of the optimum pectin extraction conditions

Pectin can be extracted from the plant material by acid, enzymes or chelating agents. However, in the case of sunflower, the pectin possesses a low amount of ester groups and is susceptible to gelling with residual calcium when exposed to elevated temperatures during processing. It is therefore essential to use chelating agents that capture divalent ions and prevent pectin from gelling during processing.

In this study, sunflower pectin was extracted with McIlvaine's buffer at different pH and different temperatures in order to determine the conditions for the highest pectin yield. The response contour plot, prepared from the extraction data, identified the conditions for optimum pectin release (> 35% pectin yield) (Figure 1).

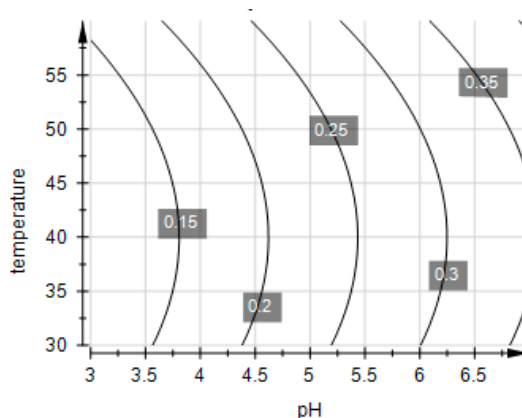


Figure 1. Two dimensional contour response plot of pectin yield as a function of pH and temperature of the extraction process. All extractions were carried out for 1 h in McIlvaine's buffer with 1:30 pulp-buffer ratio.

According to Table 3, which summarizes multiple linear regression results of pectin yields during optimization, the pectin yield was dependent on pH and increased with increasing pH, whereas the temperature was not a significant factor ($p > 0.05$). An increase in pH from 3 to 7 was accompanied by a 2-3 fold increase in pectin yield. A yield of > 35% was obtained at pH 7. Pectin release > 30% was previously obtained with 1.5% Calgon by Sabir et al. (1976), but pectin contained only 64% galacturonic acid and a high percentage of ash. The pectins obtained in this study were also 'crude' and contained buffer salts. It was therefore essential to up-scale the process and purify the pectin polymer after release.

Table 3. Multiple linear regression analysis on pectin yield, after optimization of experimental conditions ($Q^2=0.829$, $R^2=0.937$). $p=0.05$ indicates significance at the 95% level.

Parameters and interactions	Coeff. SC	P
Constant	0.23	4.88e-8
pH	0.12	5.55e-6
temp	0.02	0.09
temp*temp	0.03	0.07

3-liter-scale pectin extraction and characterization

The pectin extraction experiment was up-scaled to 3 L scale. The extraction conditions were: pH 7 and 30 °C. The yield of the purified pectin was 25.9% (w/w) on dry basis and it contained 93.6 (± 1.5) % galacturonic acid (GalA). It has been reported that pectin content in sunflower heads varies between 15 and 24%, depending on the cultivar and stage of harvest (Miyamoto, & Chang, 1992). The content of neutral sugars in the purified pectin was approx. 4%. Arabinose, galactose and rhamnose were the most abundant neutral sugars, suggesting a low content of rhamnogalacturonan I (RGI) regions in sunflower pectin (Table 4).

Table 4. Neutral sugar composition of sunflower pectin extracted in 3 liter scale.

Neutral sugar	Quantity (%)
Fucose	0.11 \pm 0.00
Rhamnose	0.84 \pm 0.00
Arabinose	1.15 \pm 0.01
Galactose	1.25 \pm 0.01
Glucose	0.39 \pm 0.00
Xylose	0.16 \pm 0.01

Fractionation and characterization of the fractions

The released pectin was fractionated by membrane filtration into the following three fractions: SPF>100 kDa, SPF50-100 kDa and SPF<50 kDa. The yields of fractionation, their degrees of methylation and acetylation and galacturonic acid contents are presented in Table 5. Fractionation resulted in two fractions very rich in galacturonic acid (SPF>100 kDa, SPF50-100 kDa), whereas SPF<50 fraction contained only approx. 40% galacturonic acid. The fractions differed in the degrees of methylation and acethylation.

Especially SPF>100 possessed much more methyl and acetyl groups than the two other fractions with lower Mws. Therefore, all three fractions represented very different pectin types.

Table 5. Yields, degrees of methylation (DM), degrees of acetylation (DAc), and galacturonic acid contents (GalA) of the three sunflower pectin fractions.

Fraction	Yield (%)	DM (%)	DAc (%)	GalA (%)
SPF<50	7.49	12.8	6.7	39.4
SPF50-100	6.35	16.1	7.4	95.5
SPF>100	86.17	39.3	10.5	97.4

The neutral sugar contents of the three fractions were low, approx. 3% in SPF<50 and SPF>100 fractions, and only 1% for the SPF50-100 fraction (Table 6). The low content of neutral sugars in SPF50-100 and SPF>100 was in agreement with the very high galacturonic acid contents present in these fractions. Apart from galacturonic acid and neutral sugars, SPF<50 probably contained other low Mw compounds that remained in the pectin after extraction. The amount of glucose in SPF<50 was much higher than in the other two fractions, which suggests cellulose degradation. The SPF<50 fraction contained no detectable rhamnose but considerable amounts of arabinose and galactose, which suggested that the abovementioned saccharides were not a part of RGI region, but existed independently in the sample. On the contrary, fractions SPF50-100 and SPF>100 fractions contained rhamnose plus arabinose and galactose, suggesting that these sugars could form RGI regions of pectin. Higher rhamnose, arabinose and galactose contents in the SPF>100 fraction indicate that the SPF>100 fraction possibly contained more RGI regions than SPF50-100 fraction.

Table 6. Mono sugar composition of sunflower pectin fractions (% w/w). Numbers in columns not sharing a letter are significantly different ($p < 0.05$).

Fraction	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Xylose
SPF<50	<0.1	<0.1	0.76±0.05 ^a	0.77±0.03 ^b	1.37±0.05 ^a	<0.1
SPF50-100	<0.1	0.23±0.01 ^b	0.19±0.01 ^b	0.33±0.02 ^c	0.25±0.02 ^b	<0.1
SPF>100	<0.1	0.84±0.04 ^a	1.03±0.15 ^a	1.14±0.12 ^a	0.2±0.07 ^b	<0.1

The average Mws of the SPF<50, SPF50-100 and SPF>100 fractions, measured by GPC (relative to standard dextrans), were 8 kDa, 16 kDa and 115 kDa, respectively (Figure 2). The differences in the retention times of the three fractions are clearly visible in the size exclusion chromatograms shown in Figure 2. The size exclusion chromatograph of SPF>100 contained one broad peak with the majority of molecules with Mws > 67 kDa. The size exclusion chromatograph of the SPF50-100 fraction showed a

narrower peak and consisted mainly of molecules between 10 and 67 kDa. The smallest fraction, SPF<50 was the most heterogeneous and contained molecules with Mws < 4.4 kDa. Mws of SPF<50 and SPF50-100 were lower than expected, when compared to the cut-off values of the membranes they were filtered through.

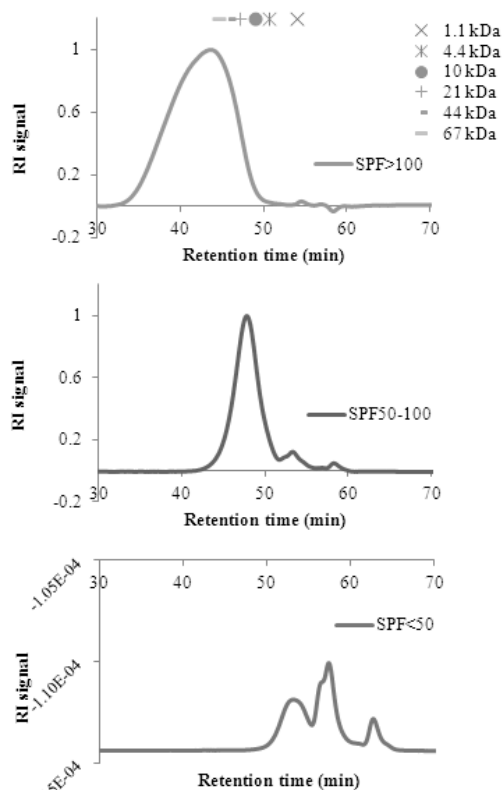


Figure 2. Size exclusion chromatograms of three sunflower pectin fractions. Lines represent the refractive index (RI) signal. Molecular weights were determined relative to a range of standard dextrans of molecular weights between 1.1 and 67 kDa.

There could be several reasons explaining this phenomenon. First of all, pectin molecules in solution have the tendency to aggregate and low ester pectins are more susceptible to aggregation than high ester pectins. Aggregated pectin polymers could be too large to pass through the membrane, even though they have relatively low Mws. Secondly, membrane systems are designed/calibrated for filtration/concentration of protein solutions. Polysaccharides in solution have larger hydrodynamic volumes than proteins of the same Mws. Therefore, a membrane with a cut-off value of e.g. 50 kDa may in fact not allow polysaccharide molecules of 50 kDa Mw to pass through the membrane.

Biological activity

Fermentation of fecal bacterial communities obtained from 5 human subjects was used to evaluate the bioactivity of three sunflower pectin fractions. QPCR was applied to measure the density of gene targets encoding 16S rRNA genes of selected bacterial taxonomic units (Fig 3).

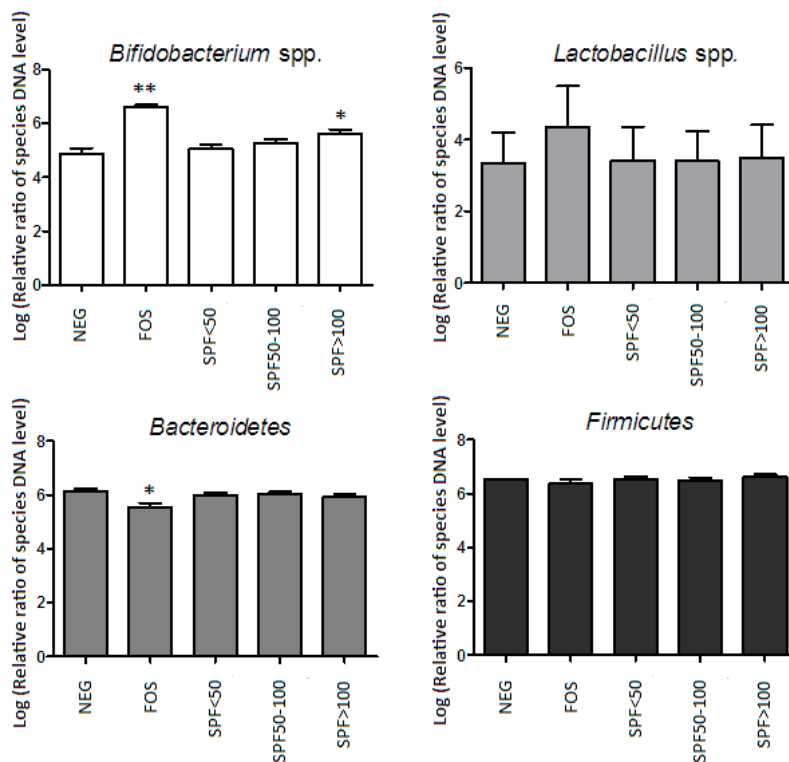


Fig 3. Relative quantities of genes encoding 16S rRNA from *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroidetes* and *Firmicutes* in fecal microbiota after fermentation of SPF<50, SPF50-100, SPF>100, FOS, and negative control (NEG). The bars represent the average response from five volunteers, whereas error bars represent SEM. Asterisks indicate a significant difference between target bacteria density in the negative control (no fibers added) and the sample fermented on particular fibers: P < 0.05, *; P < 0.01, **.

The overall composition of the intestinal microbiota in humans is dominated by *Bacteroidetes* and *Firmicutes*, hence included in the qPCR analysis. The ratio of the two phyla is supposed to be a factor in development of obesity and intestinal inflammation (Guo et al. 2008; Turnbaugh et al. 2006). The genera *Lactobacillus* and *Bifidobacterium* were included in the qPCR analysis, since they are associated with

various health benefits in the colon and hence target for prebiotic treatment (Grimoud et al., 2010; Peranet al., 2007; Shirasawa, Shibahara-Sone, Iino, & Ishikawa, 2010; Williams, 2010). Negative control (NEG) represented fermentation with no fibers added, and FOS were used as a positive control, based on their known selective effect on the content of *Bifidobacterium* and *Lactobacillus* (Palframan, Gibson, & Rastall, 2002; Rycroft, Jones, Gibson, & Rastall, 2001; Sanz, Gibson, & Rastall, 2005).

The density of *Bifidobacterium* spp. was significantly higher ($p < 0.05$) after fermentation on SPF>100 kDa compared to NEG (approx. 5 fold increase in the relative ratio of *Bifidobacterium* spp. compared to NEG) (Figure 3). The effect of SPF>100 kDa, although significant, was however minor when compared to FOS, which resulted in approx. 40 fold increase in the relative ratio of *Bifidobacterium* spp. after fecal fermentation. SPF<50 and SPF 50-100 fractions did not affect the density of *Bifidobacterium* spp. compared to NEG. Additionally, the density of *Lactobacillus* spp. was not stimulated by any of the sunflower pectin fractions.

The density of *Bacteroidetes* was significantly lower in the fecal bacterial communities fermented on FOS compared to NEG. However the three sunflower pectin fractions did not influence the density of *Bacteroidetes* after fermentation. Similarly, neither FOS nor any of the sunflower pectin fractions influenced the density of *Firmicutes*.

Discussion

Most pigments in sunflower heads are strongly associated with the pectin extract (Shi et al, 1996). Pretreatment of sunflower pulp is therefore essential to remove those dark pigments and other contaminants, such as simple sugars, proteins and minerals. Different washing media can be used for this purpose, and the type of the media used impacts the purity of the pulp and the pectin recovery after extraction. In this study, alcohol was chosen as the method for pulp washing, due to highest visible removal of pigments and lowest apparent pectin loss. Alcohol washing has the added advantage of causing partial inactivation of pectin methyl esterases present in the sunflower plant, therefore preventing natural pectin degradation (Sabir et al., 1976).

There are a few reports on the extraction of sunflower pectin in the literature. However, sunflower pectin has been previously extracted from the heads and stalks of de-seeded plants with the help of chelating agents, such as sodium hexametaphosphate (Miyamoto, & Chang, 1992; Shewfelt, & Worthington, 1953), Calgon (Sabir et al., 1976) or ammonium oxalate - oxalic acid (Bishop, 1955). The sunflower pectin

yields were dependent on the extraction conditions and ranged from 7.3%, extracted using 0.75% sodium hexametaphosphate (Miyamoto & Chang, 1992) to over 30% using 1.5% Calgon (Sabir et al., 1976). The extraction temperature was also found to be important (Chang, Dhurandhar, You, & Miyamoto, 1994; Sabir et al., 1976) and an increase from 60 to 90°C resulted in the increase of yields of oxalate- and Calgon-extracted pectins from 9 to 17%, and from 15 to 20%, respectively (Sabir et al., 1976). However, in another study, pectin yield decreased with increasing temperature (Chang et al., 1994). Our observations indicated that it was possible to obtain very high pectin yields (>30%) by extraction with McIlvaine's buffer, and that pH was the only factor that significantly influenced the pectin yield. According to the model, the temperature did not play any role in pectin release. This observation stays in agreement with the work of Chang et al. (1994), who concluded that the pH of extraction of sunflower pectin had stronger effect on the yield than temperature.

The extraction process scale up from 40 ml to 3L resulted in lower pectin yields (approx. 26%), which could be attributed to more sample handling and the dialysis step, which removed remaining buffer salts and low Mw compounds. The released pectin was very rich in galacturonic acid and contained a low amount of neutral sugars. Such a high galacturonic acid content was previously described for sunflower pectin (Chang et al., 1994; Kim et al., 1978; Lin et al., 1975; Miyamoto & Chang, 1992). The high galacturonic acid contents in the pectin sample could be due to the removal of arabinans and galactans by the alcohol washing (Kertesz, 1951).

Fractionation of sunflower pectin according to size resulted in three fractions with different properties. SPF<50 had the lowest amount of methyl and acetyl groups and a very low amount of galacturonic acid. SPF50-100 had a higher content of methyl and acetyl groups than SPF<50, galacturonic acid content >90% and a much lower amount of neutral sugars than SPF<50 and SPF>100. The last fraction, SPF>100, shared the most similarity with the 'mother' pectin, obtained after 3L extraction. It had significantly more methyl and acetyl groups and was very rich in galacturonic acid. Therefore, all three fractions were very different chemically. The methyl and acetyl contents of the pectins extracted by other authors varied from 8.4 to 38.5% (Chang et al., 1994; Iglesias, & Lozano, 2004; Miyamoto, & Chang, 1992) and from 1.4 to 13.7%, respectively (Iglesias, & Lozano, 2004; Kim et al. 1978). The literature values for methyl and acetyl contents match the results of this study and clearly indicate the low ester nature of sunflower pectin.

The Mw of sunflower pectin described in the literature varies from 39-52 kDa, measured by viscosimetry (Iglesias, & Lozano, 2004), to >523 kDa, measured by gel chromatography (Miyamoto, & Chang, 1992).

Measured Mws of sunflower pectin are affected by the extraction method, quality of the sunflower heads and the cultivar. Additionally, measured Mws are also dependent of the method of determination. Viscosimetry and gel chromatography determine Mw by measuring different polymer characteristics (hydrodynamic volume and viscosity) and neither of them give the true Mw of the polymer. Size exclusion chromatograms of the pectin fractions produced in this study revealed that the sizes of the pectin fractions did not match the cut-off values of membranes used for pectin fractionation, which could be caused by pectin aggregation or high hydrodynamic volume of polysaccharides compared to proteins of the same Mw, which are used for calibration of the cut-off value of membranes. A similar disparity in the measured Mws of biological polymers compared to membrane cut-off values was previously observed by Thomassen et al. (2011). Thomassen et al. observed that potato pulp fibers fractions of Mw between 10 and 100 kDa, had a major peak at around 5.4 kDa on a size-exclusion chromatogram.

qPCR was used to determine the abundance of target genes encoding 16S rRNA of selected bacterial taxa after fermentation in fecal slurries obtained from five subjects. The capacity of the three sunflower pectin fractions to selectively promote the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. was compared to FOS, which was used as a standard due to its well-known prebiotic effect. *Lactobacillus* spp. and *Bifidobacterium* spp. exert their probiotic effects through various mechanisms, including lowering intestinal pH, decreasing colonization by pathogenic organisms and modifying the host immune response (Williams, 2010). They are useful in preventing and treating various medical conditions, particularly those involving the gastrointestinal tract. Recent studies have demonstrated the efficacy of the use of lactobacilli and bifidobacteria in the treatment of inflammatory bowel diseases and their anti-proliferative effect on cancerous HT-29 cells (Grimoud et al., 2010), anti-inflammatory activity in the TNBS model of rat colitis (Peran et al., 2007), and suppression of *Helicobacter pylori*-induced genes in human epithelial cells (Shirasawa et al., 2010).

Additionally, the densities of *Bacteroidetes* and *Firmicutes* were also measured to evaluate the changes in the intestinal human microbiota. These two phyla dominate the human microbiota and the balance between them supposedly influences the development of obesity and intestinal inflammation (Guo et al., 2008).

Results from the *in vitro* fermentations showed selective stimulation of *Bifidobacterium* spp. by SPF>100. No other reports of the prebiotic potential of sunflower pectin can be found in the literature. However, for fibers released from potato pulp, the fraction with the largest size > 100 kDa was also the most bifidogenic (Thomassen et al., 2011). No increase in the density of bifidobacteria grown on SPF<50 and

SPF50-100 compared to NEG was observed. Additionally, none of the three fractions or FOS could stimulate the growth of *Lactobacillus* spp. compared to NEG.

None of the three sunflower pectin fractions caused significant changes in the densities of *Bacteroidetes* or *Firmicutes* after fecal fermentations. In the case of FOS, the relative ratio of *Bacteroidetes* decreased significantly ($P < 0.05$) compared to NEG, which could be due to the increase of bifidobacteria (Holck et al., 2011b). However, the increase of the *Firmicutes* to *Bacteroidetes* ratio, observed in the case of FOS, may be regarded as a negative effect because increased *Firmicutes* to *Bacteroidetes* ratio may increase the risk of obesity and influence the development of obesity-associated metabolic syndrome (Holck et al., 2011b; Ley, Turnbaugh, Klein, & Gordon, 2006; Turnbaugh et al., 2006).

Conclusion

Sunflower heads and stalks, which are rich in pectic substances, are considered agricultural wastes. Application of sunflower pectin in the food industry is very limited and restricted to utilization of its gelling ability. The results of this study provide the first positive indication for of the prebiotic potential of sunflower pectin. It has been demonstrated that sunflower pectin can be efficiently extracted with McIlvaine's buffer of pH 7 and fractionated using membrane filtration. Pectin fraction of the highest Mw, SPF>100, possessed a bifidogenic potential, when compared to negative control, whereas two other fractions, SPF<50 and SPF50-100 were not bifidogenic. None of the three sunflower pectin fractions influenced the level of *Lactobacillus* spp., *Bacteroidetes* and *Firmicutes*. Further improvements could involve purification of the oligo- and polysaccharides, derived from sunflower pulp and elucidation of the structure of the fractions to derive more conclusions about the structure-function relationships.

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5. Concluding remarks

Enzymatic catalysis for efficient release of pectin with functional properties.

Commercial cellulases possessing hydrolytic activities with respect to lime cell wall components were applied to extract pectin from lime peels. Yield and viscosity-average molecular weights of pectin polymers were dependent on extraction pH and the enzymes used. The best solution for high pectin recovery (>25% w/w) and high molecular weight (60 kDa) was achieved at pH 3.5 using the enzymes Laminex C2K or Validase TRL.

Enzymatically extracted pectin samples contained higher amounts of neutral sugars (11-15%) than classically acid-extracted pectins. Enzymatically extracted pectins were also rich in galacturonic acid (71-85% w/w). The enzymatically extracted pectin polymers were not sensitive to the presence of Ca^{2+} ions, they formed a gel at low pH in the presence of sugar and they were also able to stabilize acidified milk drinks.

Enzymatic de-esterification of pectin extracted with Laminex C2K improved its calcium sensitivity and further improved its ability to stabilize acidified milk drinks. Application of pectin methyl esterases originated from plants resulted in pectins with better stabilization abilities than pectins modified by pectin methyl esterases from fungi.

The study demonstrated that it was possible to substitute classical acid-based extraction with enzymatic catalysis and to obtain pectin products with specific functional properties.

Application of FTIR, carbohydrate microarrays and multivariate data analysis for prediction and characterization of enzyme- versus acid-extracted pectin.

FTIR spectroscopy and carbohydrate microarrays in combination with multivariate data analysis have the potential to characterize crude pectin extracts and predict pectin yields and pectin quality, including the degree of esterification of the pectin. FTIR showed especially high accuracy and precision during PLS modeling, therefore enabling the possibility for further intelligent instrumentation and online monitoring. Furthermore, simple PCA on FTIR data indicated the possibility to determine the optimal pectin extraction time without the need for interrupting the extraction process or using cumbersome, classical reference analysis techniques.

The results suggested major differences between the enzymatic and the acidically extracted crude pectin extracts with respect to the degree of esterification and abundance of RGI pectic regions. Further analysis would be necessary to determine if the detected RGI and neutral sugars belonged to the (precipitable) pectic polymer or existed free in solution.

Both FTIR and carbohydrate microarray analysis have high potential to move pectin analysis closer to pectin production, which is particularly important in industrial process and quality control.

Evaluation of the prebiotic potential of sunflower pectin fractions.

Sunflower pectin can be efficiently extracted from sunflower heads. Our observations indicate that it was possible to obtain very high pectin yield (>30%) with McIlvaine's buffer and that pH is the only factor that significantly influences pectin yield. According to the model, the temperature did not play any role in pectin release.

Fractionation of sunflower pectin according to size resulted in three fractions with different properties. SPF<50 had the lowest amount of methyl and acetyl groups and a very low amount of galacturonic acid. SPF50-100 had higher content of methyl and acetyl groups than SPF<50, galacturonic acid content >90% and much lower amount of neutral sugars than SPF<50 and SPF>100. The last fraction, SPF>100, shared the most similarity with the 'mother' pectin, obtained after 3L extraction.

Results from *in vitro* fermentations showed the selective stimulation of *Bifidobacterium* spp. only in case of SPF>100. Additionally, none of the three fractions stimulated the selective growth of *Lactobacillus* spp. and nor did FOS. None of the three sunflower pectin fractions caused significant changes in the densities of *Bacteroidetes* and *Firmicutes*. The relative increase of *Firmicutes* to *Bacteroidetes* ratio, observed in case of FOS, may potentially increase the obesity risk and influence the development of obesity-associated metabolic syndrome. It was therefore beneficial that SPF>100, having a bifidogenic potential, did not affect the ratio between the two phyla.

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