Studies on the intra- and intermolecular distributions of substituents in commercial pectins

Stéphanie Guillotin

Promotor:	prof. dr. ir. A.G.J. Voragen Hoogleraar in de levensmiddelenchemie Wageningen Universiteit
Co-promotor:	dr. H.A. Schols Universitair docent, leerstoelgroep levensmiddelenchemie Wageningen Universiteit
Promotiecommissie:	prof. dr. M.A. Cohen Stuart, Wageningen Universiteit prof. dr. ir. A.J.J. Van Ooyen, Wageningen Universiteit prof. dr. A-M. Hermansson, SIK, Göteborg, Sweden dr. C.M.G.C. Renard, INRA, Rennes, France

Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG (Voeding, Levensmiddelen-technologie, Agrobiotechnologie en Gezondheid)

Studies on the intra- and intermolecular distributions of substituents in commercial pectins

Stéphanie Guillotin

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op maandag 12 september 2005
des namiddags te vier uur in de Aula

Guillotin, Stéphanie E.

Studies on the intra- and intermolecular distributions of substituents in commercial pectins

Ph.D. thesis Wageningen University, The Netherlands, 2005
with summaries in Dutch and in French

ISBN 90-8504-265-8

Je dédie cette thèse

à mon frère

et à mes parents

Abstract

Guillotin, S.E. *Studies on the intra- and intermolecular distributions of substituents in commercial pectins*

Ph.D. thesis *Wageningen University, Wageningen, The Netherlands, 2005*

Key Words *Commerical pectins, intramolecular, intramolecular characterisation, degree of methyl-esterification, amidation, substitution, distribution of methyl-esters, amide groups*

Commercial pectins are mainly used for the gelling, thickening and stabilizing properties in food products. The different physical properties of pectins strongly depend on the galacturonic acid level and the level of methyl-esterification as well as on the molecular weight distribution. However, the conventional chemical analysis of the pectins does not always show differences between pectins while they behave differently. Two highly methyl-esterified pectins with similar chemical characteristics but different reactivity towards calcium were analysed. They were found to be a mixture of pectic populations differing in the degree of methyl-esterification as well as in the distribution of these methyl-esters. The non-calcium sensitive pectin was found to contain higher proportions of pectic populations with more random distribution of the methyl-esters but populations with a blockwise distribution of the methyl-esters were also present. These results confirm the heterogeneity of commercial pectin preparations and illustrate the need to analyse pectins on the level of (sub)populations.

Amidated pectins with similar chemical features but different calcium sensitivity were also analysed and were also found to be a mixture of different pectic populations. Methods were adapted to determine the degree of amidation and the distribution of the amide groups over the pectic backbone. The degree of substitution was different for some of the pectic populations of the commercial amidated pectins. The populations with a similar total substitution showed differences in the relative proportions of amide groups and methyl-esters as well as in the distribution of these substituents. These differences in the characteristics of the pectic populations are expected to influence the physical properties of the originating mixture as discussed for some applications.

Contents

List of abbreviations

ADD: Acid Dairy Drinks

ASRS: ultra-Self-Regenerating Anion Suppressor

BS-ir: Block Sequence Interior and/or at the Reducing end

BS-nr: Block Sequence at the Non-Reducing end

CE: Capillary Electrophoresis

CSRS: ultra-Self-Regenerating Cation Suppressor

CV: Column Volume

DAm: Degree of Amidation

DB: Degree of Blockiness

DB_{abs}: Degree of Blockiness absolute

DEAE: DiEthylAminoEthyl cellulose

DM: Degree of Methyl-esterification

DP: Degree of Polymerisation

DS: Degree of Substitution

EM: Electrophoretic Mobility

Endo-PG: Endo-PolyGalacturonase

Exo-PG: Exo-PolyGalacturonase

FTIR: Fourier Transform Infra-Red

GalA: Galacturonic Acid

GalA-nr: free Galacturonic Acid at the Non-Reducing end

GalA-ir: free Galacturonic Acid Interior and/or at the Reducing end

GC: Gas Chromatography

HM: High Methyl-esterified

HPAEC: High Performance Anion Exchange Chromatography

HPLC: High Performance Liquid Chromatography

HPSEC: High Performance Size Exclusion Chromatography

IR: Infra-Red

LM: Low Methyl-esterified

LMA: Low Methyl-esterified Amidated

MALDI-TOF MS: Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry

Mw: Molecular Weight

NMR: Nuclear Magnetic Resonance

NS: Neutral Sugar

PAD: Pulse Amperometric Detection

PME: Pectin Methyl-Esterase

PG: PolyGalacturonase

PGA: PolyGalacturonic acid

SAG: Standard Acid in Glass

(D)sap: (pectin D) saponified

$(D2)_s$: (population D2) saponified

UV: Ultra-Violet

WAX: Weak Anion Exchanger

Chapter 1

General introduction

1. Localisation of pectins, structure

1.1. History

Pectin has been discovered in the 19th century by a french scientist named Braconnot (Braconnot, 1825a; Braconnot, 1825b). He found this "acid" in so many plants that he studied the molecule and emphasised on its gelling properties. He named it "pectic acid" which is the translation of coagulum in latin. This molecule has several functional properties (e.g. gelling, thickening, emulsifying) and is widely used nowadays in food industry and in pharmaceutical products for its health effects.

1.2. Localisation

Pectins are present in almost all higher plants (Braconnot, 1825b) and in certain fresh water algae (De Vries, 1983). Pectins are mainly present in the primary wall and in the middle lamella of plant cells and they represent around 40% (dry matter basis) of the cell wall of fruits and vegetables (Brett & Waldron, 1996). In citrus fruits, they are present in several tissues at a cellular level (membranes, juice vesicules and core) in different quantities depending on the fruit variety and maturity stage (May, 1990). Pectins have a lubricating and cementing function. They are degraded during attack by plant pathogens and oligogalacturonides (ca DP 10) function as elicitors in the host-pathogen interaction (Albersheim et al., 1981).

1.3. Structure

Pectin is a complex polysaccharide composed of a α-1,4-linked D-galacturonic acid (GalA) backbone (so-called homogalacturonan or smooth region, Figures 1 and 2) and segments consisting of alternating sequences of α-(1,2)-linked L-rhamnosyl and α-1,4-linked D-galacturonosyl residues ramified with side chains of arabinans, arabinogalactans and galactans (branched rhamnogalacturonans or hairy regions) (Barrett & Northcote, 1965; Darvill, McNeill & Albersheim, 1978; De Vries, den Uyl, Voragen, Rombouts & Pilnik, 1983; De Vries, Rombouts, Voragen & Pilnik, 1982; De Vries, Rombouts, Voragen & Pilnik, 1983; De Vries, Voragen, Rombouts & Pilnik, 1981; McNeil, Darvill & Albersheim, 1980; Neukom, Amado & Pfister, 1980).

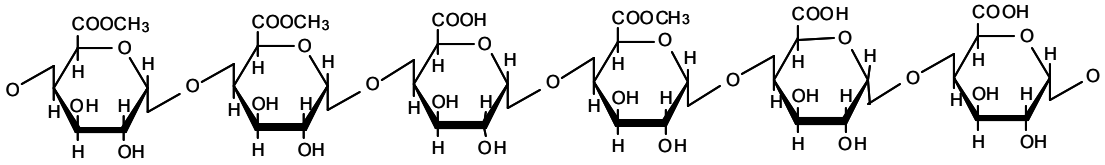

Figure 1: Homogalacturonan constituted of α-1,4-linked D-galacturonic acids.

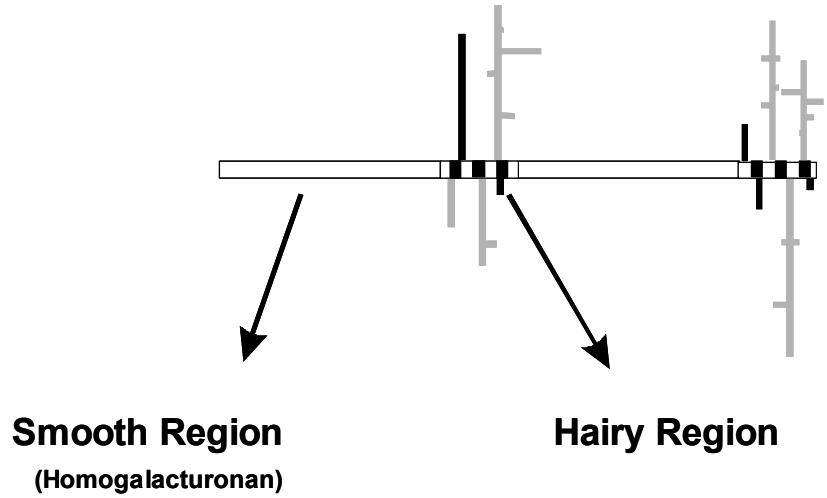

Figure 2: Pectin structure (constituted of smooth regions and hairy regions).

Other structural elements of pectins are xylogalacturonan and rhamnogalacturonan II (Figure 3). Rhamnogalacturonan II is carrying peculiar sugar residues such as Api (D-apiose), AceA (3-*C*-carboxy-5-deoxy-L-xylose), Dha (2-keto-3-deoxy-D-*lyxo*-heptulosaric acid) and Kdo (2-keto-3-deoxy-D-*manno*-octulosonic acid) (O'Neill, Ishii, Albersheim & Darvill, 2004; Vincken et al., 2003). It has been reported that the relative proportions of these different structural elements may vary significantly for different plant tissues (Voragen, Pilnik, Thibault, Axelos & Renard, 1995).

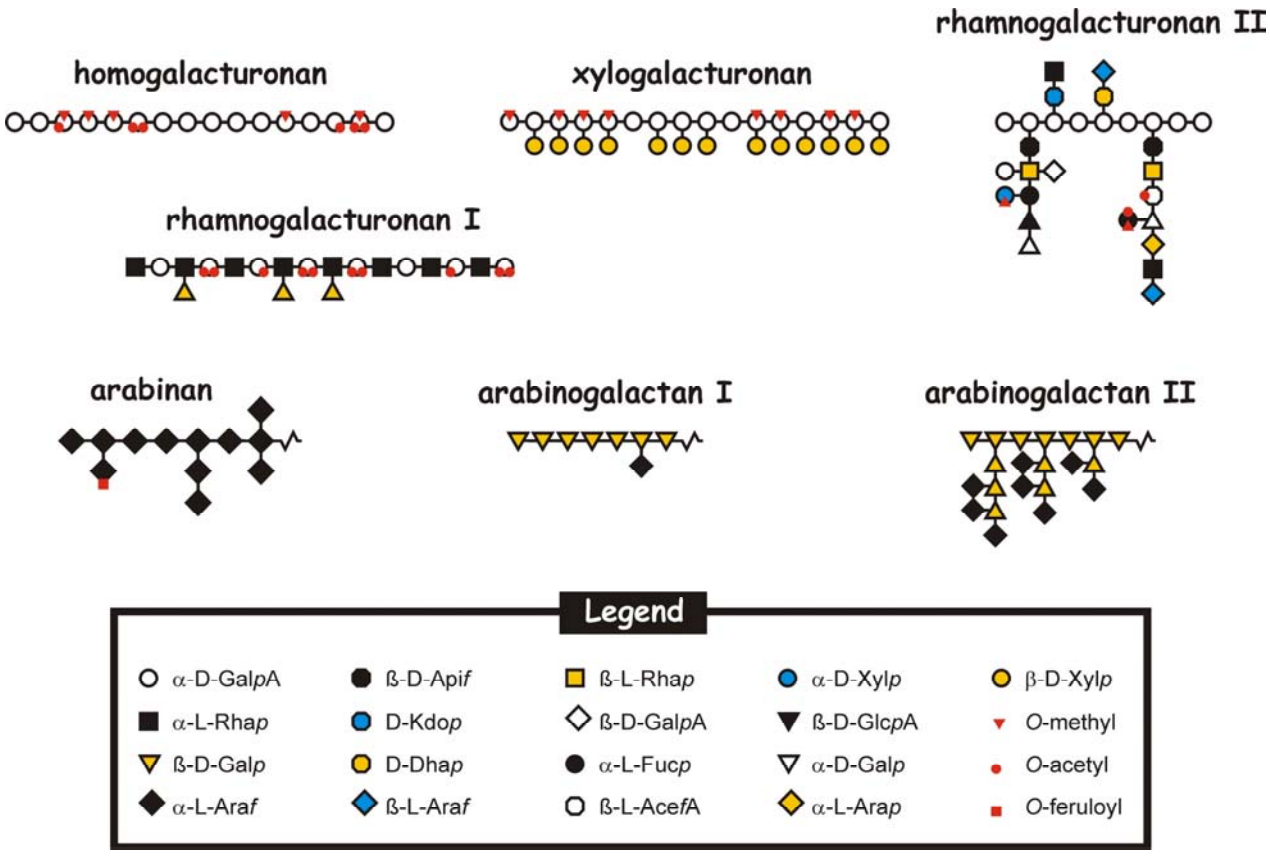

Figure 3: Different structural elements present in pectins (Vincken et al., 2003).

1.4. Commercial pectins

Pectins are used in food products for their thickening, gelling and stabilizing properties. As a result of the acid extraction, commercial pectins are essentially constituted of homogalacturonans and contain only small amounts of neutral sugars (Guillotin et al., 2005; Kravtchenko, Voragen & Pilnik, 1992a). These homogalacturonans vary from one pectin to another in function of their substituents: the GalA residues can be present as free carboxyl groups or methyl-esterified. On the positions C-2 and C-3, GalA can also be acetylated (Figure 3 and 4) such as in sugar beet and potato tuber pectins. To modify the gelling properties, HM pectins are chemically amidated as discussed later, resulting in the presence of an amide group at C-6 position of the GalA residue (Figure 4).

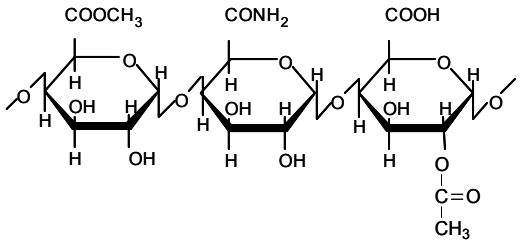

Figure 4: Representation of the different substituents potentially present in commercial pectins (respectively, methyl-ester, amide group and acetyl group).

Our study is focusing on the characterisation of commercial pectins. From high methyl-esterified pectins, methyl-ester groups can be chemically modified to amide groups (Figure 4) in the presence of ammonia in alcohol. The lower methyl-esterified amidated (LMA) pectins obtained have different physical properties compared to the methyl-esterified pectins. The physical properties of commercial pectins depend mainly on the amount and nature of the substituents (methyl-esters, acetyl or amide groups) and on the distribution of the charges over the galacturonan backbone (Lofgren, Guillotin, Evenbratt, Schols & Hermansson, 2005; Voragen et al., 1995) but also on the molecular weight (Michel, Thibault, Mercier, Heitz & Pouillaude, 1985). The methods to determine these chemical characteristics and some physical properties of the same pectins (as found by other authors) are described in more detail below.

2. Classification of commercial pectins and methods for their characterisation

Commercial pectins are mainly classified as function of their degree of methyl-esterification (DM) since it is the main parameter influencing their physical properties. The DM corresponds to the amount of moles of methanol per 100 moles of GalA.

- High Methyl-esterified pectins (HM): pectins containing 50% or more of their GalA methyl-esterified are classified as highly methyl-esterified pectins (HM). HM pectins can be further classified according to their setting time in ultra rapid set, rapid set, medium rapid set and slow set pectins (May, 1990).
- Low Methyl-esterified Non Amidated pectins (LM or LMNA): LM pectins are obtained by de-esterification of HM pectins mainly by controlling the acidity, the temperature and the time during extraction. Instead of acid, alkali can also be used to de-esterify pectins.

LM pectins obtained have less than 50% of the GalA residues methyl-esterified. The pectins possess different gelling behavior compared to HM pectins as discussed later.

- Low Methyl-esterified Amidated pectins (LMA pectins): HM pectins are chemically amidated to obtain LMA pectins with different physical properties compared to HM and LM pectins.

A short overview will be given below, on methods available to characterize pectins in detail.

2.1. Uronic acid content

The GalA content on dry basis of commercial pectins should be higher than 65% according to FAO, FCC and EU laws for food products and higher than 74% according to US Pharmacopoeia (Rolin, 2002).

Methods to determine the GalA content:

A simple titration method can be used to quantify the amount of GalA in pectins but the titration has to be corrected for the presence of substituents (methyl-esters, amide groups and acetyl groups) (Voragen et al., 1995). The GalA content can also be determined with a spectrophotometer after acid hydrolysis of pectic polymers and transformation of these monomers in furfural like compounds giving specific colours after reaction with phenol derivatives (Ahmed & Labavitch, 1977; Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979). Methyl-esters and acetyl groups have been found to interfere in the colour formation and therefore it is recommended to saponify the samples prior to their analysis. The GalA content can also be determined by HPLC after complete hydrolysis of the polymers with methanolysis or sulphuric acid hydrolysis to the constituent monomeric sugars. The monomers can then be quantified by using anion exchange chromatography (De Ruiter, Schols, Voragen & Rombouts, 1992; Verhoef et al., 2002). Infra-Red (IR) spectrometry of pectins can also be used for quantification of the GalA content (Bociek & Welti, 1975; Monsoor, Kalapathy & Proctor, 2001).

2.2. Neutral sugar content

Commercial pectins contain low amounts of neutral sugar as a result of the acid extraction. The neutral sugar (NS) content is around 5% and is constituted mainly of galactose, arabinose and rhamnose (Christensen, 1986; Guillotin et al., 2005; Kravtchenko, Voragen et al., 1992a).

Methods to determine the NS content:

The neutral sugar content of pectins can be determined after hydrolysis of the pectins in concentrated sulphuric acid by using spectrophotometric detection after reaction with phenol like reagents such as orcinol (Thibault & Robin, 1975). A more accurate method is the determination of the NS content by gas chromatography after hydrolysis of the pectins and reduction of the hydrolysed compounds into their corresponding alditol acetates (Englyst & Cummings, 1984). NS can also be quantified by using HPAEC after methanolysis, sulphuric acid or TFA hydrolysis of the samples (De Ruiter et al., 1992; Verhoef et al., 2002).

2.3. Degree of actetylation of pectins

The presence of acetyl groups results in poor gelling and thickening properties (Pippen, McCready & Owens, 1950; Ralet, Crepeau, Buchholt & Thibault, 2003) but promotes the emulsifying properties of pectins (Leroux, Langendorff, Schick, Vaishnav & Mazoyer, 2003). So far, only pectins from sugar beet, olives and potato are reported to be acetylated (May, 1990; Vierhuis, Korver, Schols & Voragen).

Methods to determine the degree of acetylation

Acetyl groups of pectin can be released by alkaline saponification and the acetic acid released in the medium is quantified by using HPLC with a resin based column (e.g Aminex HPX87H) or a reversed phase (e.g. C18) column (Levigne, Thomas, Ralet, Quemener & Thibault, 2002; Voragen, Schols & Pilnik, 1986a). The acetic acid released after saponification of the pectins can also be quantified by using a commercial acetic acid enzymatic assay kit (Chen, Schols & Voragen, 2004).

2.4. Degree of amidation

Determination of the degree of amidation of LMA pectins is important to better understand their physical behavior. By international regulation only 25% of the GalA may be substituted with amide groups in food products (Rolin & De Vries, 1990) therefore the level of amidation is limited.

Methods used to determine the degree of amidation:

To determine the DAm of LMA pectins, food industries are using the titration method (Food Chemical Codex, 1981). The drawbacks of this method are that a high amount of sample is needed and that it is rather time-consuming. IR spectrometry is also a nice tool to calculate the DAm (Sinitsya, Copikova, Prutyanov, Skoblya & Machovie, 2000) but this method can hardly be automated.

2.5. Degree of methyl-esterification

As discussed already above, the amount of methyl-esters over the pectic backbone is important for the physical properties of pectins.

Methods used to determine the DM

The degree of methyl-esterification can be determined using several methods such as titration (Food Chemical Codex, 1981), IR spectrometry (Gnanasambandam & Proctor, 2000; Haas & Jager, 1986; Reintjes, Musco & Joseph, 1962), NMR spectrometry (Grasdalen, Bakoy & Larsen, 1988). These methods are rather time consuming and can hardly be automated. Other methods using HPLC (Chatjigakis et al., 1998; Levigne et al., 2002; Voragen, Schols & Pilnik, 1986b) and GC-headspace (Huisman, Oosterveld & Schols, 2004; Walter, Sherman & Lee, 1983) analysing the methanol content after saponification of the pectins have been developed. A capillary electrophoresis method has been used a few years ago to determine the DM of the polymers as such (Jiang, Liu, WU, Chang & Chang, 2005; Jiang, Wu, Chang & Chang, 2001; Zhong, Williams, Goodall & Hansen, 1998; Zhong, Williams, Keenan, Goodall & Rolin, 1997). An advantage of the CE method is that the GalA content of the samples is not required to calculate the DM whereas the GalA values have to be known prior to the DM analysis using GC headspace and HPLC methods.

2.6. Distribution of the non-methyl-esterified GalA

Knowledge about the distribution of the charges was shown to be important in understanding the physical properties of pectins (Daas, Meyer-Hansen, Schols, De Ruiter & Voragen, 1999; Daas, Voragen & Schols, 2000; Daas, Voragen & Schols, 2001; Lofgren et al., 2005; Williams, Buffet, Foster & Norton, 2001). Citrus peels used for the extraction of pectins may contain pectin methyl-esterases (PME) which are known to de-esterify pectins in a blockwise

manner. When fungal PME is involved in de-esterification, a random distribution is obtained (Ishii, Kiho, Sugiyama & Sugimoto, 1979; Kohn, Furda & Kopec, 1968). A long storage time of the peels in conditions favourable to the action of the PME can lead to pectins having a lower DM with a much more blockwise methyl-ester distribution compared to pectins extracted from properly stored peels. In addition, de-esterification may also occur during the extraction and downstream processing of pectins. In general, under these conditions (alkaline and acid environments), pectins will be de-esterified in a random way (Daas, Meyer-Hansen et al., 1999).

Methods to determine the distribution of the methyl-esters

Since the distribution of the methyl-esters has an effect on the calcium binding, the calcium activity coefficient gives information on the distribution of the methyl-esters on the pectic backbone. In literature it is indeed reported that blocks of 7-20 free GalA residues are required for association with calcium (Braccini, Grasso & Perez, 1999; Kohn, 1975; Powell, Morris, Gidley & Rees, 1982), so pectins have stronger interaction with calcium when the DM is low and when the pectins have a blockwise distribution of the methyl-esters (Thibault & Rinaudo, 1986).

It is also possible to determine the distribution of the methyl-esters by NMR studies (Grasdalen et al., 1988). More recently, Daas et al. elaborated an enzymatic method to discriminate between pectins according to the distribution of the methyl-esters over the galacturonan backbone (Daas, Alebeek, Voragen & Schols, 1999; Daas, Arisz, Schols, De Ruiter & Voragen, 1998; Daas, Meyer-Hansen et al., 1999; Daas et al., 2000). An endo-polygalacturonase of *Kluyveromyces fragilis* degrading GalA backbone only when more than 4 adjacent non-methyl-esterified GalA units are present, is used. Subsequently, the amount of mono-, di- and trigalacturonic acid released by the enzyme is quantified by using HPAEC and the degree of blockiness is calculated from the amount of non-methyl-esterified oligomers released by the enzyme expressed as percentage of the total amount of non-methyl-esterfied GalA present in the pectin. The DB increases when the GalA residues are distributed in a more blockwise way over the pectin molecule (figure 5).

Commercial pectins were found to be a mixture of several populations (Kravtchenko, Berth, Voragen & Pilnik, 1992; Kravtchenko, Voragen & Pilnik, 1992b), therefore the distribution of the substituents can differ in an intramolecular level (within one single pectin molecule; Figure 5) or in an intermolecular level (within several pectin populations; Figure 6).

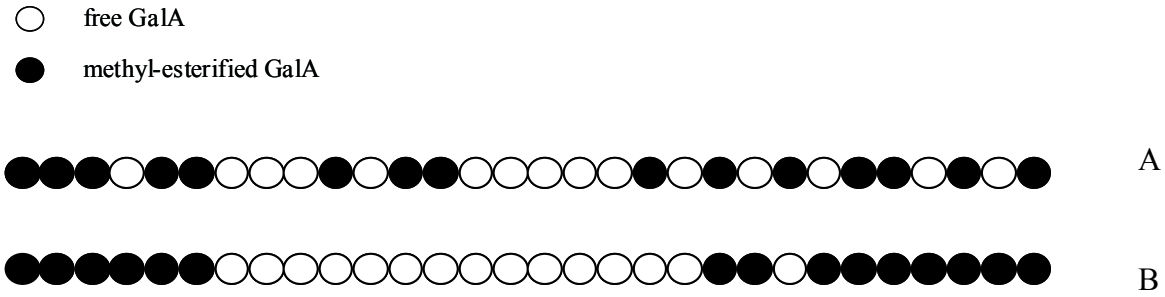

Figure 5: Same DM pectins (50%) but having different distributions of the non-methyl-esterified (free) GalA based on an intramolecular level i.e. within on single molecule. Figure A shows a random distribution while figure B shows a blockwise distribution of the non-methyl-esterified GalA.

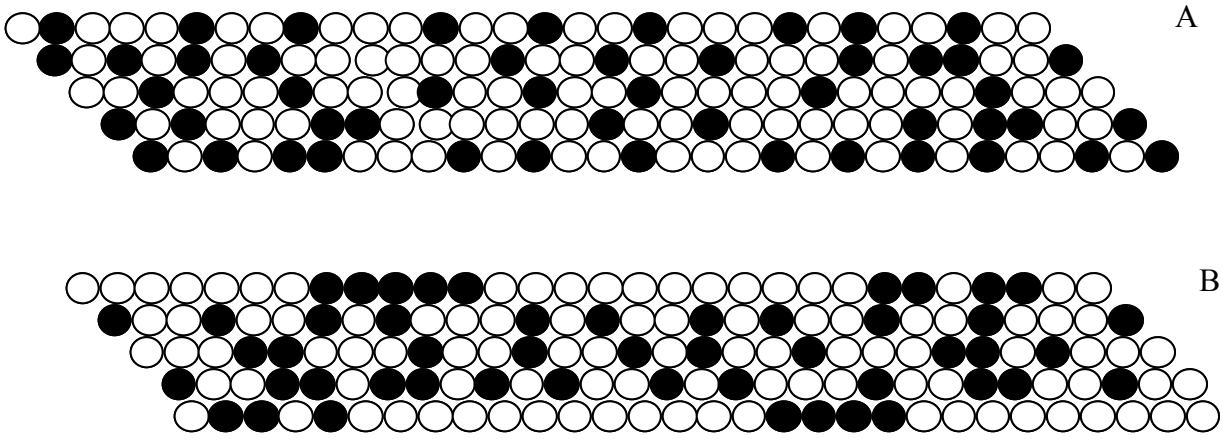

Figure 6: Schematic presentation of pectins having an overall DM of 34% but showing different intermolecular distributions of the non-methyl-esterified (free) GalA. Figure A shows a random distribution of the free GalA while Figure B shows a mixture of random and blockwise distribution of the non-methyl-esterified GalA.

Additional information on the methyl-ester distribution can be obtained with a more detailed analysis of the mono-, di- and triGalA and (partially) methyl-esterified oligomers released after endo-polygalacturonase attack. A higher amount of triGalA illustrates the presence of longer endo-PG degradable sequences (Daas, Boxma, Hopman, Voragen & Schols, 2001; Daas et al., 2000). Apart from the DB and the comparison of the proportion of mono-, di- and triGalA molecules released, a third parameter can be determined: the ratio of oligomers without methyl-esters versus the amount of oligomers carrying methyl-esters. The higher this ratio, the more closely associated blocks are present (Daas, Boxma et al., 2001; Daas et al.,

2000). This ratio thus provides more information on the distribution of non-methyl-esterified blocks.

The distribution of the substituents of LMA pectins is more complex to study compared to methyl-esterified pectins as a result of their substitution with both amide groups and methyl-esters. Controversial results have been found for the distribution of amide groups since some authors suggested a blockwise distribution of the amide groups (Racape, Thibault, Reitsma & Pilnik, 1989; Racape, Thibault, Reitsma & Pilnik, 1987) (with calcium activity coefficients studies) while others found a random distribution of these groups (enzymatic studies and ion exchange separation) (Anger & Dongowski, 1988; Voragen, Schols, Clement & Pilnik, 1984). This controversy may also be due to differences in the method used to study the distribution of the substituents or even in the preparation of the amidated samples studied.

2.7. Molecular weight

The physical properties of pectins strongly depend on the molecular weight. Higher molecular weights of the pectins lead to a stronger gel (Christensen, 1954; Owens, Svenson & Schultz, 1933; Van Deventer-Schriemer & Pilnik, 1987). In the case of oil-water emulsions, it has been reported that the surface tension is reduced when the degree of polymerization is decreased, probably due to a faster kinetic of these low molecular weight molecules to the interface (Leroux et al., 2003).

Methods to determine the molecular weight:

The molecular weight (Mw) of pectins is difficult to determine and is the source of many debates. HPSEC (High Performance Size Exclusion Chromatography) using pectins to calibrate the system has been widely used in food industry to determine the Mw of pectins. This method is fast but the separation depends on the shape of the pectins (hydrodynamic volume) rather than the molecular weight (Mw). Accurate molecular weight measurement is possible only when the molecules analysed have the same molecular shape and density as the standards used (Kravtchenko, Voragen et al., 1992a). Since the hydrodynamic volume of pectins depends on the degree of methyl-esterification of pectins (Kravtchenko, Berth et al., 1992) and/or to the degree of branching with neutral sugars (Kravtchenko, Voragen et al., 1992a), the HPSEC is not always an accurate method.

To optimise the Mw analysis, HPSEC can be coupled to an on-line viscosity detector although the intrinsic viscosity is also related to the hydrodynamic volume (Corredig, Kerr & Wicker, 2000). Pectins eluting from the size exclusion columns can also be analysed with light scattering detection, but the drawback of this method is that pectins can form aggregates perturbing the light scattering detection. Prior to analysis, the aggregates have to be removed by filtration. The average Mw of pectins estimated in literature is varying from 140 up to 225 kDa (Corredig & Wicker, 2001; Lecacheux & Brigand, 1988; Morris, Foster & Harding, 2000; Yoo, Fishman, Hotchkiss & Lee, 2005) although much higher values can be found.

3. Sources and extraction of commercial pectins

3.1. Source of pectins

Pectins are present in almost all higher plants. Several by-products of the food industries are used for their extraction, such as citrus peels (by-product of lemon juice production), apple pommace (by-product of apple juice manufacture), sugar beet (by-product of the beet-sugar industry) and in a minor extend potatoes fibres, sunflower heads (by-product of oil production) and onions (May, 1990).

3.1.1. Extraction of pectins

Extraction of pectins has to be fast to avoid degradation of pectins in the raw materials by enzymes produced by micro-organisms (PME, PG, PL etc) or by native PME present in the raw material (May, 1990). The degradation of pectins during storage of the source materials by enzymes may lead to pectins with completely different gelling behavior. To avoid this, raw materials have to be dried immediately after production.

3.1.1.1. HM pectins

HM pectins are extracted from the pomace or peels in hot diluted mineral acid at pH1-3 at 50-90 ºC during 3-12 hours (Rolin, 2002). Dry citrus peels contain 20 to 30% of pectin on a dry matter basis, lower amounts are present in dried apple pomace (10 to 15%) (Christensen, 1986). By adding alcohol (usually isopropanol but methanol or ethanol are also used) the pectins are precipitated. Finally, the gelatinous mass is pressed, washed, dried and ground

(May, 1990). Depending on the process conditions, pectins with a DM from 55 to 80% are obtained (Rolin, 2002).

3.1.1.2. LM pectins

To produce other types of pectins, esters can be hydrolysed by the action of acid or alkali either before or during an extraction, as concentrated liquid or in the alcoholic slurry before separation and drying. When alkali is used the reaction has to be performed at a low temperature and in aqueous solutions to avoid β-eliminative degradation of the polymers (Kravtchenko, Arnould, Voragen & Pilnik, 1992). LM pectins can also be extracted with aqueous chelating agents such as hexametaphosphate (e.g. potato pectins) (Voragen et al., 1995). The use of PME for the production of LM pectins can be an alternative for the chemical extraction (Christensen, 1986). The low methyl-esterified pectins obtained can form gels in the presence of calcium at a higher pH range compared to HM pectins as described later.

3.1.1.3. LMA pectins

The acid de-esterification process in order to obtain LM pectin is time consuming and the gel formation is not easy to control with LM pectins. Therefore a new process has been set up: the amidation of HM pectins. Pectins can be amidated in heterogeneous phases (in the presence of water/alcohol/ammonia) (Anger & Dongowski, 1988) but also in homogeneous phases (concentrated aqueous ammonia) (Black & Smit, 1972). The amidated pectins obtained are used in other applications than the methyl-esterified pectins since they have different physical properties (Black & Smit, 1972).

3.1.1.4. Acetylated pectins

Since the second world war, pectins have been extracted from sugar beet residues. These pectins are not of a very high quality in terms of gelation due to a lower Mw of these pectins, the presence of a considerable amount of acetyl groups, a higher NS content and consequently a lower GalA content. Treatment in acidic methanol removes the acetyl groups and increases the level of methyl-esters but this treatment also decreases the Mw significantly. The GalA content is even often below the limit permitted by regulations (Rolin, 2002). However, acetylated pectins are used for their emulsifying properties (Leroux et al., 2003).

4. Physical behavior of pectins

The gelling behavior of pectins depends on several parameters as described above (GalA, degree of substitution, nature of the substituents, Mw). It is also important to know the pKa of pectins to understand their gelling behavior according to the pH: the pKa value is in the range of 3.5-4.5 (Plaschina, Braudo & Tolstoguzov, 1978; Ravanat & Rindaudo, 1980; Rolin, 2002).

4.1. HM pectins

HM pectins are generally used at low pH (2.5-3.8) with high sugar content (around 55%) but without calcium addition (May, 1990; Voragen et al., 1995). The low pH used for gelling decreases the charge repulsions while the presence of sugar reduces the water binding (Voragen et al., 1995). The speed of setting of the gels is determined by the DM. To obtain a wide range of gelling properties pectin preparations (from different sources) can be blended but generally they are chemically modified by de-esterification or amidation as described above. The mechanism of the gel formation is still unclear but there is some evidence that in the junction zones of such gel, hydrophobic bonds between methyl-ester groups are involved as well as hydrogen bonds (Lapasin & Pricl, 1995). The nature of the sugar co-solute (e.g. glucose or fructose) as well as its concentration are very important (May, 1990). The HM pectin gels are not thermo-reversible (Rolin & De Vries, 1990).

4.2. LM pectins

LM pectins are used mainly in the presence of calcium within a wide pH range (2.8-7). It was also shown that LM pectins can gel at acidic pH (1.6) without calcium (Gilsenan, Richardson & Morris, 2000; Voragen et al., 1995). LM pectins gels are thermoreversible (Rolin & De Vries, 1990). They are believed to gel by the “egg box” mechanism (De Vries, Rombouts et al., 1983) first suggested for alginates (Clark & Ross-Murphi, 1987). Sections of two pectic chains, which must be free of ester groups, are held together by a number of calcium ions (Figure 7). It is reported that blocks of 7-20 free GalA residues are required for association with calcium (Braccini et al., 1999; Kohn, 1975; Powell et al., 1982). The texture of LM pectin gels can be adjusted by controlling the calcium to pectin ratio. A high pectin content with relatively low amounts of calcium will give an elastic gel, while the use of more calcium

with a minimum of pectin will produce a much more brittle (fragile) product, possibly with syneresis. All these different parameters make LM pectins very versatile thickeners and gelling agents.

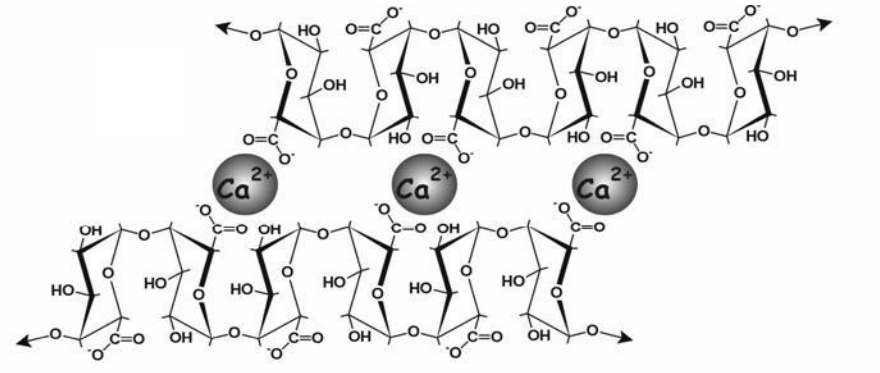

Figure 7: Gelling mechanism of LMA pectins in the presence of calcium (egg box model).

4.3. LMA pectins

The pH range of gel formation of LMA pectins is similar to the one of LM pectins (pH 2.8-7). LMA pectins can gel in a wider range of calcium (10-80 mg/g of pectin) compared to LM pectins (20-40 mg/g pectin) (Christensen, 1986; May, 1990). The natural calcium content of the fruit is generally sufficient to enable gel formation in the case of jam preparation. LMA gels are also claimed to be perfectly thermoreversible (Racape et al., 1989) and the firmness and the strength of LMA gels in the presence of calcium are higher compared to gels of methyl-esterified pectins with the same degree of substitution (Black & Smit, 1972). The gelling mechanism of amidated pectins is not completely understood yet. It seems that both the egg-box mechanism described previously for LM pectins and stabilization of the junction zones by the hydrogen bonds of amide groups play an important role (Alonso-Mougan, Meijide, Jover, Rodriguez-Nunez & Vazquez-Tato, 2002).

4.4. Acetylated pectins

The gelling performance of acetylated pectins is very limited due to the acetyl content (Pippen et al., 1950) and it has been shown that acetyl groups hinder dimerisation of pectins through calcium ions (Ralet et al., 2003). The high NS content and low Mw of acetyl pectins is also a disadvantage for the gel formation (Dea & Madden, 1986; Michel et al., 1985; Phatak, Chang & Brown, 1988). Nevertheless, acetylated pectins have several interesting properties. One of

their advantage is the ability of their gel to be dehydrated and rehydrated (May, 1990). Sugar beet pectins also carry ferulic acid residues, ester linked to arabinosyl or galactosyl residues of neutral sugar side chains. These ferulate monomers can be coupled into dehydrodimers by treatment with hydrogen peroxide/peroxidase or ammonium persulfate and this mechanism increases the viscosity and gelling of beet pectins (Thibault, Garreau & Durand, 1987). The gel formation of acetylated pectins in the presence of calcium can as well be improved after enzymatic treatment with pectin acetyl esterase besides pectin esterase (Oosterveld, Beldman, Searle-van Leeuwen & Voragen, 2000). Finally, acetylated pectins are important for their good emulsifying ability compared to non-acetylated pectins (Leroux et al., 2003). It is suggested in literature that beet pectins are able to reduce the interfacial tension between an oil phase and a water phase resulting in efficient emulsion. Acetyl groups of the pectins may play a role by reducing the calcium bridging floculation (Leroux et al., 2003) or by enhancing the hydrophobicity of pectins (Dea & Madden, 1986).

4.5. Use of pectins in food products and drinks

Since decades, food industries spend time and money to improve food products or to innovate new products in texture, taste and appearance. Several gelling agents such as carragenan, alginate, guar, xanthan, gelatin, starch and pectin are used to change the texture of food material. These main hydrocolloids are used in different applications since their gelling and thickening properties depend on the conditions of the product (pH, presence of co-solute, salts and temperature). Pectins are mainly extracted from fruits and are thus natural gelling agents. As natural product and due to their different physical properties, pectins are widely used for several food systems: jams, marmelades, dairy drinks, dessert (fillings in bakery products), candies, salad dressing, fruit and tomato pastes (Braddock, 1999). Pectic acid and short chains of polygalacturonic acid (at pH 5,5) can be used as clarification agents to precipitate the cloudiness of fruit juices (Braddock, 1999). In dairy drinks, pectins can be used to stabilise cloud (Voragen et al., 1995). The different types of pectins can be used in different applications. HM pectins are used in high sugar products such as jams (above 60% soluble solids). They can also be used in dairy products since they prevent aggregation of casein on heating at a pH below 4.3 e.g. in the case of UHT (ultra-high-temperature)-treated drinkable yoghurts (May, 1990). With the increase of low calorie products on the market due to the awareness of the consumers of their weight, reduced sugar jams of $\approx$ 30% soluble solids or

lower are produced using LM pectins. LMA pectins can be used for bakery purposes (such as fillings for cakes) since their gels are thermally reversible (they will melt and reset to a good gel on cooling). Glazes for pastries, flans, low sugar content yogurts with fruits addition are also made with amidated pectins. LMA pectin gels have also less tendency to give syneresis (Rolin, 2002). LMA pectins can gel under the same conditions as the HM pectins and at lower temperature as the methyl-esterified ones with the same amount of charges (Rolin, 2002). Amidation improves the gelling properties of low esterified pectins (May, 1990).
Some syneresis problems may occur in jams and this cannot always be avoided using a different type or amount of pectin, a different pH or a different soluble solid or calcium content. An alternative can be the addition of neutral gums but the drawback is the flavour decrease of the product (May, 1990).

Standardisation of the gelling power of pectins

Pectin characteristics depend on several external factors such as the fruit variety, the ripening conditions and the availability of the raw material, which is fluctuating on the market. Pectin manufacturers therefore standardize pectins by mixing different batches of pectins or by mixing the pectin with sucrose (up to 50% of sucrose is allowed; Rolin, 2002).
To determine the gelling power of HM pectins the SAG (standard acid in glass) value is determined. Boiled pectin solutions with sugar added are poured in a standardized jelly glass containing a precalculated amount of acid. After mixing, a gel forms on cooling to 25 °C after 20-24 hours. The gels are removed from the glass by turning it upside down and the sagging of the gel under its own weight after 2 minutes standing is measured. This value corresponds to the gel strength and is converted to a 'Jelly Grade" of the pectin (May, 1990).

5. Aim and outline of the thesis

Pectin manufacturers are still not able to predict conveniently the physical properties of commercial pectins. Some pectins have similar chemical features whereas the gelling behavior is quite different.
The aim of this thesis was to broaden our knowledge of the fine structure of commercial pectins used as ingredients in the food industry to better understand their technical functionality. For this reason, the research focussed on the distribution of galacturonosyl residues with free carboxyl groups in HM, LM and amidated pectins taking into account the

heterogeneity of pectin preparations. Methods had to be developed to establish the heterogeneity of pectin preparations, to fractionate these preparations in sub-populations and to adapt and further develop the approach of Daas et al. (Daas, Alebeek et al., 1999; Daas et al., 1998; Daas, Meyer-Hansen et al., 1999; Daas et al., 2000) to further characterise pectins, in particular amidated pectins.

The approach followed in this thesis was to analyse the samples with similar chemical characteristics and to develop new methods to detect differences on a molecular level and to clarify the link between their structure and their physical properties. So far, the distribution of the free GalA was analysed on crude commercial samples (Daas, Boxma et al., 2001; Daas, Meyer-Hansen et al., 1999; Daas et al., 2000; Limberg et al., 2000). Pectins are known to be heterogeneous with respect to their charge (Kravtchenko, Voragen et al., 1992b; Schols, Reitsma, Voragen & Pilnik, 1989). Our study focussed on the study of the pectin populations fractionated from commercial pectins to obtain more information about the gelling behavior as function of the fine chemical structure and to explain unclear behavior of commercial pectin preparations with very similar chemical specifications. These pectic populations were separated on anion exchange chromatography and characterised. Since amidated pectins have not been studied extensively in the past, amidated samples were included in this research to analyse the distribution of substituents.

We first aimed to find a rapid method to differentiate pectins using anion exchange HPLC (chapter 2). HM pectins with similar chemical characteristics and different behavior in application have been fractionated by preparative anion exchange chromatography (chapter 3) to study the features of these pectic populations in detail. We also included amidated pectins in our research. Since available methods to determine the degree of amidation are limited, we first adapted a method using capillary electrophoresis (CE) to analyse the degree of amidation of the samples and compare the results with the results obtained using FTIR and titration methods (Chapter 4). Finally, the distribution of amide groups has been investigated using enzymatic digestion and analysis of the oligomers with CE (chapter 4) and HPAEC at pH5 (Chapter 5). Two LMA pectins with similar chemical characteristics but different gelling behavior were fractionated and the fractions were characterized with respect to the distribution of substituents (Chapter 6). Chapter 7 discusses the relation between pectin structure and the physical properties.

References

Ahmed A. E. R., Labavitch J. M. (1977). *Journal of Food Biochemistry*, *1*, 361-365.

Albersheim P., Darvill A. G., McNeil M., Valent B. S., Han M. G., Lyon G., Sharp J. K., Desjardins A.-E., Spellman M. W., Ross L. M., Robertson B. K., Aman P., Fransen L. E. (1981). *Pure and Applied Chemistry*, *53*, 79.

Alonso-Mougan M., Meijide F., Jover A., Rodriguez-Nunez E., Vazquez-Tato J. (2002). Rheological behaviour of an amide pectin. *Journal of Food Engineering*, *55*, 123-129.

Anger H., Dongowski G. (1988). Amidated pectins - characterization and enzymatic degradation. *Food Hydrocolloid.*, *2*, (5), 371-379.

Barrett A. J. B., Northcote D. H. (1965). Apple fruit pectic substances. *Biochemical Journal*, (94), 617-627.

Black S. A., Smit C. J. B. (1972). The effect of demethylation procedures on the quality of low-ester pectins used in dessert gels. *Journal of Food Science*, *37*, (II), 730-732.

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

Bociek S., Welti D. (1975). The quantitative analysis of uronic acid polymers by infrared spectroscopy. *Carbohydrate Research*, *42*, 217-226.

Braccini I., Grasso R. P., Perez S. (1999). Conformational and configurational features of acidic polysaccharides and their interactions with calcium ions: a molecular modeling investigation. *Carbohydrate Research*, *317*, 119-130.

Braconnot H. (1825a). Nouvelles observations sur l'acide pectique. *Annales de chimie et de physique*, *30*, 96-102.

Braconnot H. (1825b). Recherches sur un nouvel acide universellement répandu dans tous les végétaux. *Annales de chimie et de physique*, *28*, 173-178.

Braddock R. J., (1999). In: *Handbook of citrus by-products and processing technology*; Wiley, J. & Sons, Inc, New-York.

Brett C., Waldron K., (1996). In: *Physiology and biochemistry of plant cell walls*. Cambridge.

Chatjigakis A. K., Pappas C., proxenia N., Kalantzi O., Rodis P., Polissiou M. (1998). FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes. *Carbohydrate Polymers*, *37*, 395-408.

Chen Z., Schols H. A., Voragen A. G. J. (2004). Differently sized granules from acetylated potato and sweet potato starches differ in the acetyl substitution pattern of their amylose populations. *Carbohydrate Polymers*, *56*, 219-226.

Christensen P. E. (1954). Methods of grading pectin in relation to the molecular weight (intrinsec viscosity) of pectin. *Food Research*, *19*, 163.

Christensen S. H. (1986). Pectins. *Food Hydrocolloids*, *3*, 205-230.

Clark A. H., Ross-Murphi S. B. (1987). Structural and mechanical properties of biopolymer gels. *Advances in Polymer Science*, *83*, 57-192.

Corredig M., Kerr W., Wicker L. (2000). Molecular characterisation of commercial pectins by separation with linear mix gel permeation columns in-line with multi-angle light scattering detection. *Food Hydrocolloids*, *14*, 41-47.

Corredig M., Wicker L. (2001). Changes in the molecular weight distribution of three commercial pectins after valve homogenization. *Food Hydrocolloids*, *15*, 17-23.

Daas P. J. H., Alebeek G. J. W. M. v., Voragen A. G. J., Schols H. A., (1999). Determination of the distribution of non-esterified glacturonic acid in pectin with endo-polygalacturonase. In: *Gums and Stabilisers for the food industry*; Williams P. A., Phillips G. O. eds, Wrexham, The Royal Society of Chemistry, p3-18.

Daas P. J. H., Arisz P. W., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1998). Analysis of Partially Methyl-Esterified Galacturonic Acid Oligomers by High-Performance Anion-Exchange Chromatography and Matrix-Assisted laser Desorption/Ionization Time-of Flight Spectrometry. *Analytical Biochemistry*, *257*, 195-202.

Daas P. J. H., Boxma B., Hopman A. M. C. P., Voragen A. G. J., Schols H. A. (2001). Nonesterified Galacturonic Acid Sequence Homology of Pectins. *Biopolymers.*, *58*, 1-8.

Daas P. J. H., Meyer-Hansen K., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research*, *318*, 135-145.

Daas P. J. H., Voragen A. G. J., Schols H. A. (2000). Characterisation of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research*, *326*, 120-129.

Daas P. J. H., Voragen A. G. J., Schols H. A. (2001). Study of the Methyl Ester Distribution in Pectin with Endo-polygalacturonase and High Performance Size exclusion Chromatography. *Biopolymers*, *58*, 195-203.

Darvill A. G., McNeill M., Albersheim P. (1978). Structure of plant cell walls: VIII. A new pectic polysaccharide. *Plant Physiology*, 62, 418-422.

De Ruiter G. A., Schols H. A., Voragen A. G. J., Rombouts F. M. (1992). Carbohydrate analysis of water-soluble uronic acid containing polysaccharides with high-performance anion-exchange using methanolysis combined with TFA hydrolysis is superior to four other methods. *Analytical Biochemistry*, 207, 176-185.

De Vries J. A., (1983). Structural features of apple pectic substances. Ph.D Thesis, Wageningen Agricultural university, The Netherlands.

De Vries J. A., den Uyl C. H., Voragen A. G. J., Rombouts F. M., Pilnik W. (1983). Structural features in the neutral sugar side chains of apple pectic substances. *Carbohydrate Polymers*, 6, 193-205.

De Vries J. A., Rombouts F. M., Voragen A. G. J., Pilnik W. (1982). Enzymatic degradation of apple pectins. *Carbohydrate Polymers*, 2, 25-33.

De Vries J. A., Rombouts F. M., Voragen A. G. J., Pilnik W. (1983). Distribution of methoxyl groups in apple pectic substances. *Carbohydrate Polymers*, *3*, 245-258.

De Vries J. A., Voragen A. G. J., Rombouts F. M., Pilnik W. (1981). Extraction and purification of pectins from alcohol insoluble solids from ripe and unripe apples. *Carbohydrate Polymers*, *1*, 117-127.

Dea I. C. M., Madden J. K. (1986). Acetylated pectic polysaccharides of sugar beet. *Food Hydrocolloids*, *1*, 71-88.

Englyst H. N., Cummings J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst*, *109*, 937-942.

Food Chemical Codex, (1981) 3rd Ed., National Academy of Sciences, Washington, DC.

Gilsenan P. M., Richardson R. K., Morris E. R. (2000). Thermally reversible acid-induced gelation of low-methoxy pectin. *Carbohydrate Polymers*, *41*, 339-349.

Gnanasambandam R., Proctor A. (2000). Determination of pectin degree of esterification by diffuse refelectance Fourier transform infrared spectroscopy. *Food Chemistry*, *68*, 327-332.

Grasdalen H., Bakoy O. E., Larsen B. (1988). Determination of the degree of esterification and the distribution of methylated and free carboxyl groups in pectins by h-nmr spectroscopy. *Carbohydrate Research*, 184, 183-191.

Guillotin S. E., Bakx E. J., Boulenguer P., Mazoyer J., Schols H. A., Voragen A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*, 391-398.

Haas U., Jager M. (1986). Degree of esterification of pectins determined by photoacoustic near infrared spectroscopy. *Journal of Food Science*, *51*, (4), 1087-1088.

Huisman M. M. H., Oosterveld A., Schols H. A. (2004). New method for fast determination of the degree of methylation of pectins by headspace GC. *Food Hydrocolloids.*, *18*, (4), 665-668.

Ishii S., Kiho K., Sugiyama S., Sugimoto H. (1979). Low-Methoxyl Pectin Prepared by Pectinesterase from *Aspergillus japonicus*. *Journal of Food Science*, *44*, 611-614.

Jiang C. M., Liu S.-H., WU M.-H., Chang W.-M., Chang H.-M. (2005). Determination of the degree of esterification of alkaline de-esterified pectins by capillary zone electrophoresis. *Food Chemistry*, *91*, 551-555.

Jiang C. M., Wu M. C., Chang W.-H., Chang H.-M. (2001). Determination of random- and blockwise-type de-esterified pectins by capillary zone electrophoresis. *Journal of Agricultural and Food Chemistry*, *49*, 5584-5588.

Kohn R. (1975). Ion binding on polyuronates. Alginate and pectin. *Pure and Applied Chemistry*, *42*, 371-397.

Kohn R., Furda I., Kopec Z. (1968). Distribution of free carboxyl groups in the pectin molecule after treatment with pectin esterase. *Collection of Czechoslovak Chemical Communications*, *33*, 264-269.

Kravtchenko T. P., Arnould I., Voragen A. G. J., Pilnik W. (1992). Improvement of the selective depolymerisation of pectic substances by chemical beta-elimination in aqueous solution. *Carbohydrate Polymers*, *19*, 237-242.

Kravtchenko T. P., Berth G., Voragen A. G. J., Pilnik W. (1992). Studies on the intermolecular distribution of industrial pectins by means of preparative size exclusion chromatography. *Carbohydrate Polymers*, *18*, 253-263.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992a). Analytical comparison of three industrial pectin preparations. *Carbohydrate Polymers*, *18*, 17-25.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992b). Studies on the intermolecular distribution of industrial pectins by means of preparative ion-exchange chromatography. *Carbohydrate Polymers*, *19*, 115-124.

Lapasin R., Pricl S., (1995). In: *Rheology of industrial polysaccharides: theory and applications*; London, UK.

Lecacheux D., Brigand G. (1988). Preparative fractionation of natural polysaccharides by size exclusion chromatography. *Carbohydrate Polymers*, *8*, 119-130.

Leroux J., Langendorff V., Schick G., Vaishnav V., Mazoyer J. (2003). Emulsion stabilizing properties of pectin. *Food Hydrocolloids*, *17*, 455-462.

Levigne S., Thomas M., Ralet M. C., Quemener B., Thibault J.-F. (2002). Determination of the degrees of methylation and acetylation of pectins using a C18 column and internal standards. *Food Hydrocolloids*,

Limberg G., Korner R., Buchholt H. C., Christensen T. M. I. E., Roepstorff P., Mikkelsen D. J. (2000). Quantification of the amount of galacturonic acid residues in blocksequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger*. *Carbohydrate Research*, *327*, 321-332.

Lofgren C., Guillotin S., Evenbratt H., Schols H., Hermansson A.-M. (2005). Effects of calcium, pH and blockiness on kinetic rheological behavior and microstructure. *Biomacromolecules*, *6*, 646-652.

May C. D. (1990). Industrial pectins: sources, production and applications. *Carbohydrate Polymers*, *12*, 79-99.

McNeil M., Darvill A. G., Albersheim P. (1980). Structure of plant cell walls. X. Rhamnogalacturonan I, a structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. *Plant Physiology*, *66*, 1128-1134.

Michel F., Thibault J.-F., Mercier C., Heitz F., Pouillaude F. (1985). Extraction and characterization of pectins from sugar beet pulp. *Journal of food science*, 50, 1499-1502.

Monsoor M., Kalapathy U., Proctor A. (2001). Determination of polygalacturonic acid content in pectin extracts by diffuse reflectance Fourier transform infrared spectroscopy. *Food Chemistry*, *74*, 233-238.

Morris G. A., Foster T. J., Harding S. E. (2000). The effect of the degree of esterification on the hydrodynamic properties of citrus pectin. *Food Hydrocolloids*, *14*, 227-235.

Neukom H., Amado R., Pfister M. (1980). Neuere erkenntnisse auf dem gebiete der pektinstoffe. *Lebensm.wiss.u technology*, 13, 1-6.

O'Neill M. A., Ishii T., Albersheim P., Darvill A. G. (2004). Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review of Plant Biology*, *55*, (1), 109-139.

Oosterveld A., Beldman G., Searle-van Leeuwen M. J. F., Voragen A. G. J. (2000). Effect of enzymatic deacetylation on gelation of sugar beet pectin in the presence of calcium. *Carbohydrate Polymers*, *43*, 249-256.

Owens H. S., Svenson H. A., Schultz T. H., (1933). In. *Natural Plant Hydrocolloids, Advances in Chemistry;* Washington DC: Ameridan Chemical Society, 10.

Phatak L., Chang K. C., Brown G. (1988). *Journal of Food Science*, *53*, 830-833.

Pippen E. L., McCready R. M., Owens H. S. (1950). *Journal of the American Chemical Society*, *72*, 813-816.

Plaschina I. G., Braudo E. E., Tolstoguzov V. B. (1978). Circular dicroism studies of pectin solutions. *Carbohydrate Research*, *60*, (1),

Powell D. A., Morris E. R., Gidley M. J., Rees D. A. (1982). Conformations and interactions of pectins II. Influence of residue sequence on chain association in calcium pectate gels. *Journal of molecal biology*, 155, 517-531.

Racape E., Thibault J. F., Reitsma J. C. E., Pilnik W. (1989). Properties of amidated pectins II. Polyelectrolyte behavior and calcium binding of amidate pectins and amidated pectic acids. *Biopolymers*, *28*, 1435-1448.

Racape E., Thibault J.-F., Reitsma J. C. E., Pilnik W. (1987). Preparation and characterisation of amidated pectic acids, *Food Hydrocolloids*, 1, (5/6), 571-572.

Ralet M. C., Crepeau M. J. C., Buchholt H. C., Thibault J.-F. (2003). Polyelectrolyte behaviour and calcium binding properties of sugar beet pectins differing in their degrees of methylation and acetylation. *Biochemical Engineering Journal*, *3735*, 1-11.

Ravanat G., Rindaudo M. (1980). Investigation on oligo- and polygalacturonic acids by potentiometry and circular dichroism. *Biopolymers*, *19*, 2209-2222.

Reintjes M., Musco D. D., Joseph G. H. (1962). J*ournal of food sciences*, *27*, 441-445.

Rolin C., (2002). Commercial pectin preparations. In: *Pectins and their Manipulation*; Seymour G. B., Knox J. P., Blackwell Publishing Ltd, 222-239.

Rolin C., De Vries J. A., (1990). Pectin. In: *Food gels*; Harris, P.J., 401-434.

Schols H. A., Reitsma J. C. E., Voragen A. G. J., Pilnik W. (1989). High-performance ion exchange chromatography of pectins. *Food Hydrocolloids*, *3*, (2), 115-121.

Sinitsya A., Copikova J., Prutyanov V., Skoblya S., Machovie V. (2000). Amidation of highly methoxylated citrus pectin with primary amines. *Carbohydrate Polymers*, *42*, 359-368.

Thibault J.-F. (1979). Automatisation du dosage des substances pectiques par la méthode au meta-hydroxydiphenyl. *Lebensmittel Wissenschaft und Technologie*, *12*, 247-251.

Thibault J.-F., Garreau C., Durand D. (1987). Kinetics and mechanism of the reaction of ammonium persulfate with ferulic acid and sugar-beet pectins. *Carbohydrate Research*, *163*, 15-27.

Thibault J.-F., Rinaudo M. (1986). Chain association of pectic molecules during calcium-induced gelation. *Biopolymers*, *25*, 456-468.

Thibault J.-F., Robin J.-P. (1975). Automatisation du dosage des acides uroniques par la méthode au carbazol. Application au cas des matières pectiques. *ann. techno. agric.*, *24*, (I), 99-110.

Van Deventer-Schriemer W. H., Pilnik W. (1987). Studies on pectin degradation. *Acta Alimentaria*, *16*, 143.

Verhoef R., de Waard P., Schols H. A., Rätto M., Siika-aho M., Voragen A. G. J. (2002). Structural elucidation of the EPS of slime producing *Brevundimonas vesicularis* sp. isolated from a paper machine. *Carbohydrate Research*, *337*, 1821-1831.

Vierhuis E., Korver M., Schols H. A., Voragen A. G. J. Structural characteristics of pectic polysaccharides from olive fruit *(Olea europaea cv Moraiolo)* in relation to processing for oil extraction. *Carbohydrate Polymers*, *51*, 135-148.

Vincken J.-P., Schols H. A., Oomen R. J. F. J., McCann M. C., Ulvskov P., Voragen A. G. J., Visser R. G. F. (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology*, *132*, 1781-1789.

Voragen A. G. J., Pilnik W., Thibault J.-F., Axelos M. A. V., Renard C. M. G. C., (1995). Pectins. In: *Food polysaccharides and their applications;* Stephen A. M., New York: Marcel Dekker Inc, 287-339.

Voragen A. G. J., Schols H. A., Clement A. J. J., Pilnik W., (1984). Enzymic analysis of pectins. In: *Gums and stabilisers for the food industry.2. Applications of Hydrocolloids;* Philips G. O., Wedlock D. J., Williams P. A., ed, Elsevier London, 517-521.

Voragen A. G. J., Schols H. A., Pilnik W. (1986a). Analysis of the degree of methylation and acetylation of pectins by hplc. *Food Hydrocolloids*, *1*, (1), 65-70.

Walter R. H., Sherman R. M., Lee C.-Y. (1983). A comparison of methods for polyuronide methoxyl determination. *37*, 12.

Williams M. A. K., Buffet G. M. C., Foster T. J., Norton I. T. (2001). Simulation of endo-PG digest patterns and implications for the determination of pectin fine structure. *Carbohydrate Research*, *334*, 243-250.

Yoo S. H., Fishman M. L., Hotchkiss A. T., Lee H. G. (2005). Viscometric behavior of high-methoxy and low-methoxy pectin solutions. *Food Hydrocolloids*,

Zhong H. J., Williams M. A. K., Goodall D. M., Hansen M. E. (1998). Capillary electrophoresis studies of pectins. *Carbohydrate Research*, *308*, 1-8.

Zhong H. J., Williams M. A. K., Keenan R. D., Goodall D. M., Rolin C. (1997). Separation and quantification of pectins using capillary electrophoresis. *Carbohydrate Polymers*, *32*, (1), 27-32.

Chapter 2

Rapid HPLC method to screen pectins for heterogeneity in methyl-esterification

To be submitted in Food Hydrocolloids as
S.E. Guillotin, A. Van Loey, P. Boulenguer, H.A. Schols and A. G. J. Voragen.

Abstract

Functionality of pectins as a food ingredient is strongly related to their chemical fine structure. Chemical characteristics of pectins are determined by many different parameters in their manufacture (choice of the raw material and extraction conditions). Pectin companies are thus in need for rapid methods to check the performance of extracted pectins. An important factor in the characterisation is the homogeneity of the pectin preparation, which is usually determined by laborious, time consuming, soft gel based chromatographic procedures. A rapid method using a weak anion exchange column (WAX column) to screen commercial pectins prior to fractionation on preparative scale is presented and exemplified with the rapid analysis of pectins having different levels and distributions of methyl-ester groups. Amidated pectins were also included in the study.

1. Introduction

Pectins are mainly used in food industries for their gelling and stabilizing properties. For industrial applications, they are usually extracted from lemon peels and apple pomaces. Traditionally, they are used as gelling agents in jams, jellies and marmalades to compensate for the lack of pectin in the fruits themself but they are also used in confectionery, bakery fillings and milk acid products (May, 1990; Rolin, 2002).

Pectins are complex mixtures of polysaccharides composed of a galacturonan backbone (homogalacturonan or so-called smooth region) of which variable proportions can be methyl-esterified (Barrett & Northcote, 1965; De Vries, Voragen, Rombouts & Pilnik, 1981). In addition, so-called hairy regions are present, constituted of alternative sequences of rhamnose and galacturonic acid (rhamnogalacturonan I) carrying neutral side chains (arabinans, arabinogalactans) attached to the rhamnose moieties (Darvill, McNeill & Albersheim, 1978; McNeill, Darvill & Albersheim, 1980; Neukom, Amado & Pfister, 1980; Pilnik & Voragen, 1991; Voragen, Pilnik, Thibault, Axelos & Renard, 1995). Next to these structural elements, three other elements have been found in pectins: xylogalacturonan, apiogalacturonan, rhamnogalacturonan II (O'Neill, Ishii, Albersheim & Darvill, 2004; Vincken et al., 2003). As a result of the acid extraction, commercial pectins are rich in GalA (> 70%, w/w) and contain only small amounts of neutral sugars (5-10%, w/w) (Guillotin et al., 2005; Kravtchenko, Voragen & Pilnik, 1992a; Lecacheux & Brigand, 1988).

Depending on the degree of methyl-esterification (DM), pectins are classified as high methyl-esterified (HM) pectins or as low methyl-esterified non amidated (LM) pectins. HM pectins can also be chemically amidated to obtain low methyl-esterified and amidated (LMA) pectins. HM pectins are used mainly in sugar-acid gels whereas LM and LMA pectins are used in pectate gels. Both gelling and stabilizing properties are influenced by the molecular weight (Christensen, 1954; Owens, Svenson & Schultz, 1933; Van Deventer-Schriemer & Pilnik, 1987), the level and distribution of methyl-esters (Lofgren, Guillotin, Evenbratt, Schols & Hermansson, 2005; Rolin, 2002; Thibault & Rinaudo, 1986; Voragen et al., 1995). Differences in gelling behavior of pectins with almost identical chemical characteristics could be attributed to differences in the distribution of methyl-esterified carboxyl groups over the pectic backbone. Differences in the methyl-ester distribution can be observed within one pectic molecule (intramolecular level) or between different molecules (intermolecular level). The gelling properties of commercial samples are complex to study since it has been shown

with anion exchange chromatography (Guillotin et al., 2005; Kravtchenko, Berth, Voragen & Pilnik, 1992; Ralet & Thibault, 2002; Schols, Reitsma, Voragen & Pilnik, 1989) or size exclusion chromatography (Kravtchenko, Berth et al., 1992; Ralet, Bonnin & Thibault, 2001) that they are not homogenous but constituted of several pectic populations with different chemical features. These populations also showed variations in the amount of methyl-esters (Kravtchenko, Berth et al., 1992; Kravtchenko, Voragen & Pilnik, 1992b; Schols et al., 1989) and in the distribution of the substituents as it was recently shown for LM and HM pectins after elution on Source-Q anion exchanger (Guillotin et al., 2005). However, using anion exchange chromatography on a "soft" gel (DEAE-sepharose CL-6B) and conductometric characterization, Ralet & Thibault (2002) were not able to see an effect of the methyl-ester distribution. These contradictory results may be due to the different anion exchanger used leading to different separation mechanisms. Both chromatographic methods used are conventional semi-preparative separations that require high amounts of samples and take long elution times (~6 hours).

There is a need for a rapid analytical screening procedure to analyze pectins. Schols et al. (1989) were able to separate pectic populations present in commercial pectins according to their charges, using an HPLC system equipped with an anion exchange column (MA7P column) on an analytical scale. This method was much less time consuming compared to the earlier methods performed with conventional ion exchange chromatography using DEAE columns (Anger & Dongowski, 1984; Heri, Neukom & Deuel, 1961). Since the MA7P column used by Schols et al. (1989) is not available anymore, other anion-exchange columns have been tested in order to find an alternative column able to fractionate pectins in the same way. Samples with different levels and distributions of methyl-esters and amide groups have been used to examine the potential of a Dionex Propac WAX-10 column (WAX-10) in the rapid analysis of pectins.

2. Experimental

2.1. Samples

Pectins C56 and C67 (Copenhagen pectin A/S; Lille Skensved, Denmark) used in this study and pectins M93, M85, R70, CR52 and CR31 obtained after demethyl-esterification or methyl-esterification of pectin C67 were characterised in detail in the study of Daas et al. (Daas, Meyer-Hansen, Schols, De Ruiter & Voragen, 1999).

The pectin M93 has then been treated in our study with tomato PME or *Aspergillus aculeatus* PME. The pectin (4 mg/ml) was incubated either with tomato PME (0.19 U/ml in 0.1M Tris-HCl pH 8) at 55°C or *Aspergillus aculeatus* PME (0.14 U/ml in 0.1 M Na-Acetate pH 4.5) at 50 °C. Pectins are mixed 3 min with the enzyme and incubated for 7, 13, and 18 min. The enzyme was inactivate at 80°C during 2 min. Pectins were obtained with a DM of 79, 71 60 and 53% with Aspergillus PME (Asp79, Asp71, Asp60, Asp 53) and a DM of 81, 75, 70 and 66% with tomato PME (Tom81, Tom75, Tom70 and Tom66). Two samples were included as control: pectin M93 without enzyme in buffer solution at pH 4.5 and 8. All the samples were ultrafiltrated (Millipore filter device, 10 kDa) and diluted in 15 mM phosphate buffer pH 6 prior to injection on a Propac^{TM} WAX-10 (WAX) column.

Pectins A and B were kindly provided by Degussa Texturant Systems (Baupte, France). In a previous study, they were also called respectively Calcium Sensitive (CS) pectin and Non Calcium Sensitive (NCS) pectin. Their different physical properties in the presence of calcium have been studied (Laurent & Boulenguer, 2003). Both pectins A and B were obtained from lemon peel (same citrus variety) but using a different process and were selected for having nearly the same molecular weight (82 and 78kDa, respectively), degree of methyl-esterification (74% and 72%, respectively) and galacturonic acid content (82 and 74 w/w %, respectively) (Guillotin et al., 2005). The neutral sugar (NS) content is low for both pectins (7 and 12 w/w %, respectively). The low methyl-esteried and amidated (LMA) pectins D and G were also from Degussa Texturant Systems and were selected for their different physical properties in the presence of calcium. Pectins D and G have the same Mw (≈ 73 kDa), similar DM (29 and 31%, respectively), degree of amidation (DAm) (19 and 18%, respectively) and GalA content (68 and 70 % w/w, respectively).

2.2. Analytical methods

2.2.1. Chromatographic analysis of pectins on analytical scale

An Akta purifier system equipped with an A-900 autosampler (Amersham Biosciences) was used for the separation of pectins on a Dionex Propac^{TM} WAX-10 column (WAX; 250 × 4 mm). After an equilibration step of 10 min (1 ml/min) with "Millipore" water, 200 µl of pectin solution (5 mg/ml) was injected (pectin powder was wetted in ethanol prior to solubilisation in water). Elution (1 ml/min) was performed with a linear gradient from 0 to 0.6

M of sodium phosphate buffer (pH 6) in 15 min and the gradient was hold at 0.6M sodium phosphate (pH 6) for 25 min. At the end of the gradient, the column was washed for two min with "Millipore" water and was then eluted with 0.1 M sodium hydroxide for 8 min. Detection was accomplished with an UV detector (Amersham Biosciences) set at 215 nm. The baseline of all elution patterns were corrected by using the baseline obtained upon injection of 200 µl of water.

2.2.2. Uronic acid and neutral sugars contents

The uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl method (Ahmed & Labavitch, 1977; Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979). Total neutral sugars were estimated with the automated orcinol method (Tollier & Robin, 1979), using galactose as a standard.

2.2.3. Degree of methyl-esterification

The amount of methanol formed after saponification of the pectins was analysed by using a colorimetric method (Klavons & Bennett, 1986). In this method, methanol is oxidized to formaldehyde with alcohol oxidase, followed by the condensation of the formaldehyde with 2,4-pentanedione to the colored product 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (Wood & Siddiqui, 1971). This colored product is determined with a spectrophotometer at 412 nm. Alcohol oxidase from *Pichia pastoris* with an activity of 25 units/mg (EC 1.1.3.13) was purchased from Sigma. One unit will oxidize 1 µmol of methanol to formaldehyde per minute at pH 7.5 and 25°C (Sigma). Triplicates were analysed and the average methanol concentration was calculated.

3. Results and discussion

3.1. Separation of commercial pectins on WAX column

Since the experimental MA7P column used by Schols et al. (1989) was not further commercialised, this rapid HPLC method never found application in pectin analysis. In order to find an alternative column able to fractionate pectins in the same way, other anion-exchange columns have been tested. Technical problems occurred with the anion exchange

mini Q column (Amersham Biosciences; 30 × 3.2 mm) due to the high back pressure of the column during the gradient. These pressure problems may be due to the interactions of the viscous polymeric pectin solutions with the matrix of the mini-Q column. A weak anion exchange column (WAX) was found to be able to separate pectins (Figure 1) comparable with the results of Schols et al. (1989) without pressure problems and rather high resolution between the different pectic populations. A background correction is necessary to correct for the increase in UV absorption due to the phosphate buffer during the gradient making the pectin populations clearly visible. The first peak eluting at 1.5 min corresponded to the elution of high DM pectin not bound to the column (M85 and M93, Figure 1B).

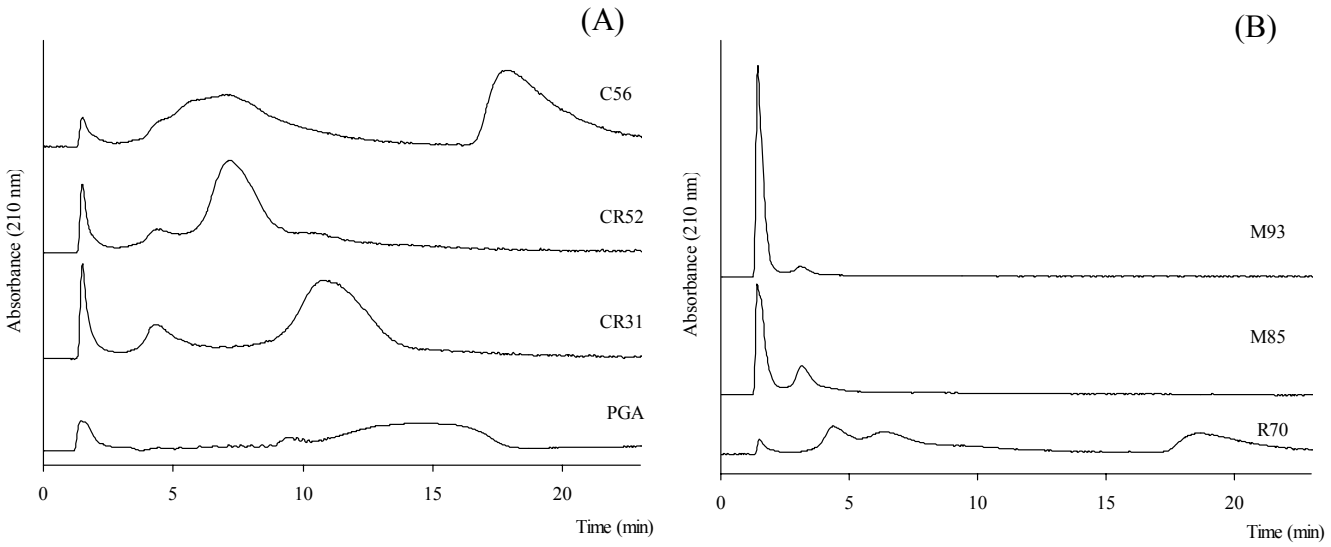

Figure 1: WAX elution profiles after background correction of different DM pectins well characterized (Daas et al., 1999). Polygalacturonic acid (PGA), commercial extracted pectin (C56) and commercial random de-esterified pectins (CR31, CR52) are shown in Figure A, random de-esterified pectins and highly methyl-esterified pectins (R70, M85, M93) are shown in Figure B. The arabic number corresponds to the degree of methyl-esterification.

However, also neutral carbohydrates will not be retained by the anion exchanger as demonstrated for a commercial DM 70 pectin (Figure 2). The NS content of commercial pectins is low (5-10% in w/w) and in the same range as the values mentioned previously for commercial lemon pectins (Guillotin et al., 2005; Kravtchenko, Voragen et al., 1992a; Lecacheux & Brigand, 1988). Part of these neutral sugars may have been released during the acid hydrolysis of the peel/pomace to extract pectins and not completely removed in the further pectin isolation process. Such neutral sugars would indeed not bind to the anion

exchanger. As the commercial pectins are similarly low in NS content, the proportion of high DM pectins eluting at 1.9 min can be estimated with a negligible error. Polygalacturonic acid (PGA) is the most negatively charged pectin totally free of methyl-esters and consequently is eluted at the end of the gradient (broad peak eluted from 10-16.5 min). When commercial pectins were analysed (C56 and pectin B, Figure 1A and 2), an additional peak was observed around 18 min, even later than PGA. This peak was found to contain negligible amounts of galacturonic acid (GalA) and neutral sugars (Figure 2). It consisted of "impurities" present in the pectin sample which could be removed by an ethanol wash (results not shown). Obviously, pectins were eluted from the WAX column according to the DM as was found by Schols et al. (1989) with the MA7P column. Furthermore, it was interesting to notice that the two commercial pectins with similar DM (CR52 and C56) presented several populations in different relative amounts (Figure 1A) illustrating the heterogeneity of these pectins.

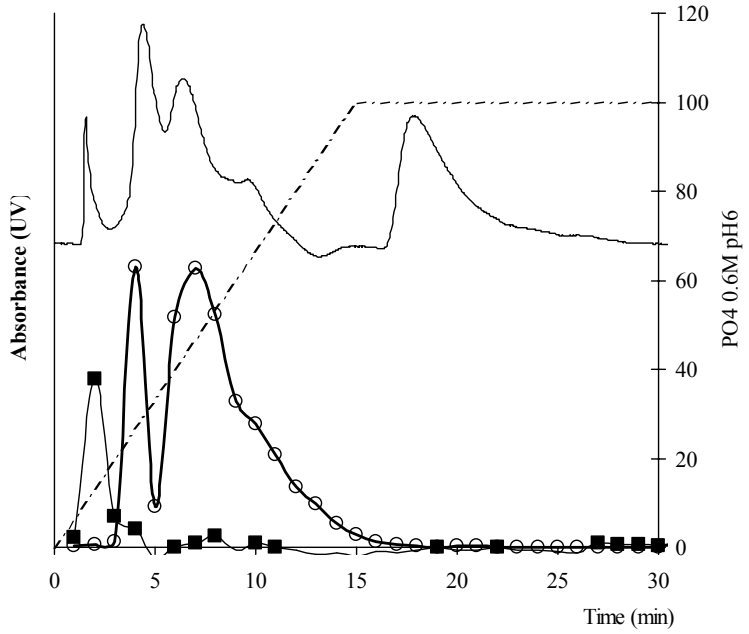

Figure 2: elution profiles after background correction of the commercial pectin B analysed on WAX column. (-) UV 210 nm, (○) Uronic acid content , (■) NS content.

3.2. Elutions profiles of commercial HM pectins with similar chemical characteristics but different physical properties

The above-described method was used to investigate the elution behavior of two commercial HM pectins (A and B) originating from the same citrus variety but showing totally different gelling properties in the presence of calcium (Laurent & Boulenguer, 2003). The GalA content and DM could not explain the differences in physical properties since these characteristics were rather similar for the pectins A and B. The intra- and intermolecular charge distribution of these two samples might however be different and therefore the samples were analysed on the WAX column. Similar to previously analysed commercial samples also for pectins A and B, a non-sugar containing peak around 18 min could be observed. The elution patterns of both pectins A and B showed several populations, which were found to differ in the peak ratio (Figure 3). Also more pectic populations were observed for pectin B (four main populations) compared to pectin A (three main populations).

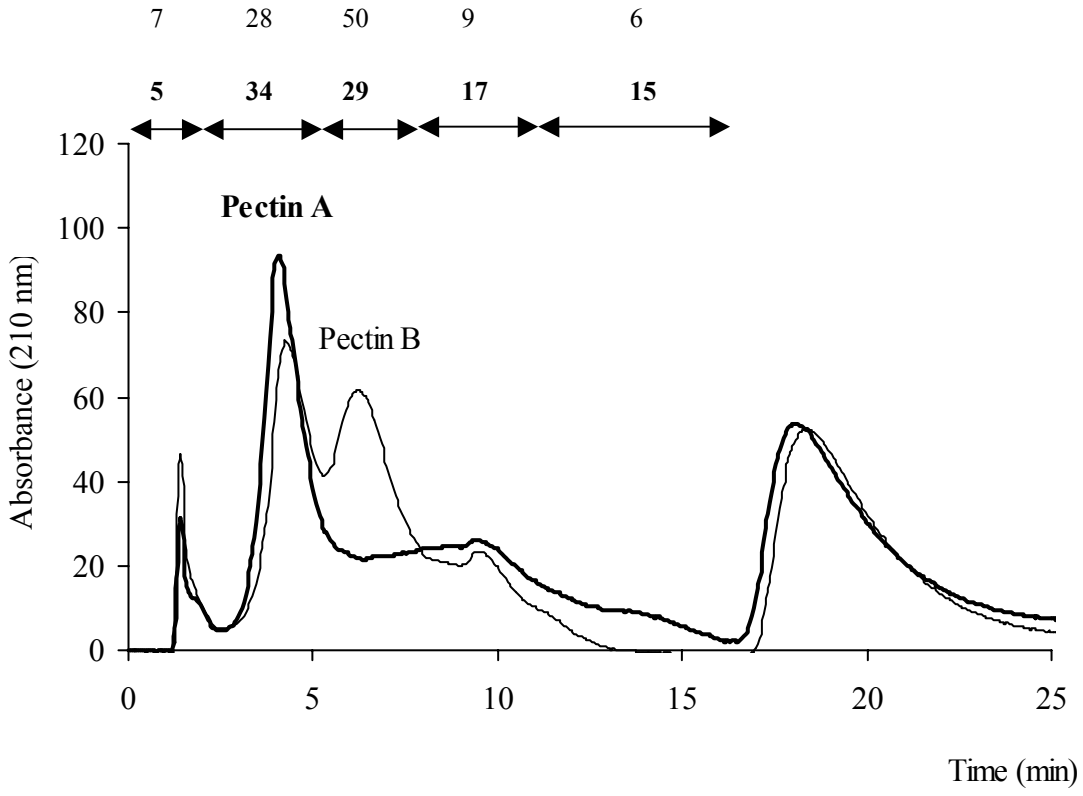

Figure 3: WAX elution profiles after background correction of two commercial HM pectins A and B. The arabic numbers indicate the peak area of pectin B and the bold arabic numbers indicate the peak area of pectin A (% of the total peak area).

It is also interesting to notice that 32% of the pectin A was eluting with a high ionic (between 9-15 min) strength whereas only 15% of the pectin B was eluting under these conditions. The different populations in pectins A and B may account for the observed differences in gelling properties. We further aimed to verify the possibility that peaks eluting under similar conditions may be different in terms of DM and methyl-esters distribution. Next to a detailed characterisation of the individual populations also the mechanism for the different elution behavior of the various pectic sub-fractions was subject for further studies (Guillotin et al., 2005). However, to be able to characterise the sub-populations, high amounts of samples were needed and therefore commercial pectins were fractionated on a preparative Source-Q anion exchange column resulting in similar elution patterns as obtained on the WAX column. It has been shown that commercial pectins were heterogeneous and the pectic populations were differing not solely according to the total charge (DM) but also according to the charge density (degree of blockiness of free carboxyl groups over the pectic backbone) (Guillotin et al., 2005).

3.3. Elutions profiles of commercial LMA pectins with similar chemical characteristics but different physical properties

Low methyl-esterified amidated pectins were also included in this study. Two commercial LMA pectins (D and G) were selected since they showed different physical properties in the presence of calcium despite similar chemical characteristics.

Pectin D was found to be more calcium reactive compared to pectin G. Rather small differences in chemical composition (GalA content, DM and degree of amidation) were observed. Elution patterns were shown to be slightly different (Figure 4). One of the differences in the WAX profile of the pectins was the presence of the peak eluting at high ionic strength (~ 18 min) although it did not contain GalA nor NS and can be removed with ethanol as shown previously. This observation was surprising since both pectins were submitted to the same extraction process including ethanol wash. Another difference was the intensity of the peak at 9 min elution time compared to the peak at 13 min elution time. The peak area ratio of those 2 peaks was lower for pectin D than for pectin G (respectively 5.4 and 7.9) indicating less variation in the proportion of these two populations for pectin D. These differences may explain some of the physical behavior observed for pectins D and G.

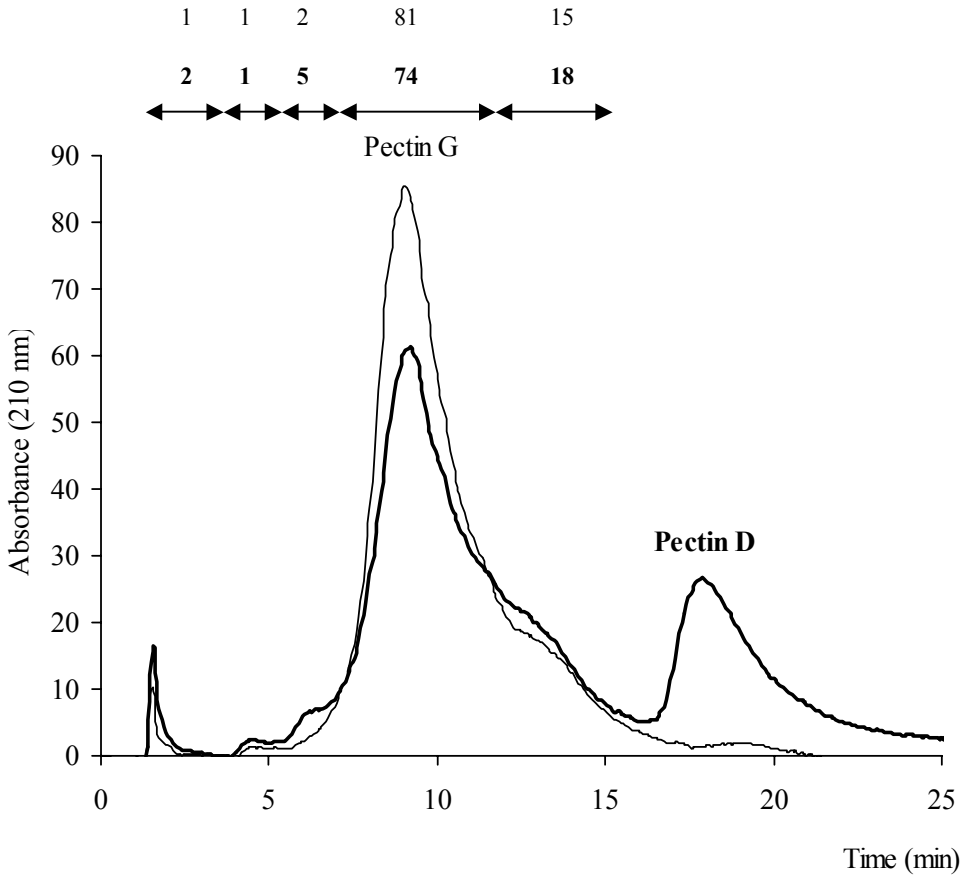

Figure 4: WAX elution profiles after background correction of two commercial LMA pectins D and G. The arabic numbers indicate the peak area in percentage compared to the total peak area of the pectic populations.

3.4. Separation of pectins with blockwise and random distribution of methyl-esters

The WAX column was also checked for its ability to monitor changes in DM and methyl-ester distribution changes introduced by chemical or enzymatic de-esterification. These characteristics can be modified using pectin methyl-esterase (PME): plant PME (such as tomato PME) is known to de-esterify pectin by a blockwise mechanism, whereas fungal PME (such as *Aspergillus aculeatus* PME) de-esterifies pectins in a random fashion (Ishii, Kiho, Sugiyama & Sugimoto, 1979; Kohn, Furda & Kopec, 1968). Different degrees of methyl-esterification (M80 to DM50) were obtained starting from the same DM 93 pectin and using tomato PME (at pH 8) and *Aspergillus aculeatus* PME (at pH 4.5). For each DM and depending on the enzyme used, pectins with a random or blockwise methyl-ester distribution were obtained. For pectins de-esterified in a random way, the binding to the column increased

with lower DM (Figure 5) as it has been observed previously on the MA7P column (Schols et al., 1989). From the profiles, it can be seen that the high DM population was de-esterified first by the fungal PME: it represented 59% of the total peak area for the DM93 and only 2% of the total peak area for the DM53 as indicated in figure 5. The second pectic population eluting at 2.1 min was less modified.

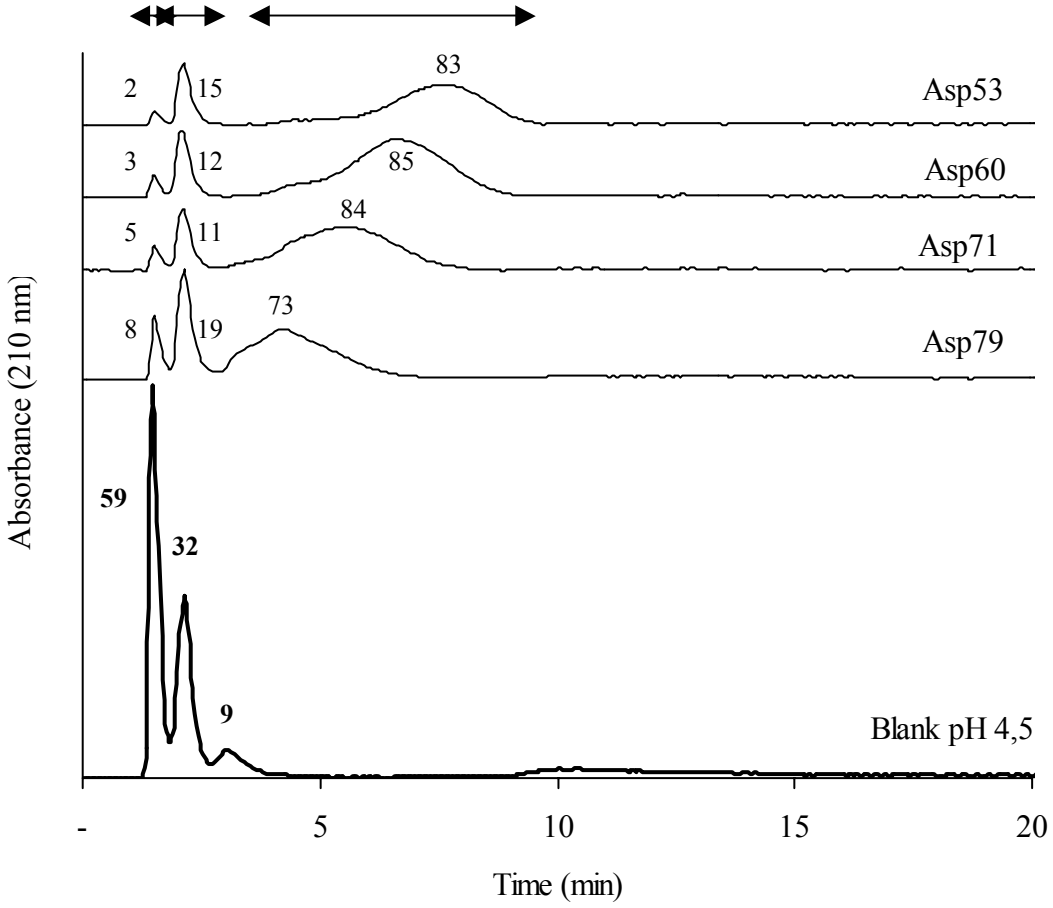

Figure 5: WAX elution profiles after background correction of de-esterified pectins with *Aspergillus aculeatus* PME with a DM of 53% (Asp53), 60% (Asp60), 71% (Asp71), 79% (Asp79) and pectin M93 (at pH 4.5) without enzyme (Blank pH 4.5). The arabic numbers indicate the peak area in percentage compared to the total peak area.

The WAX column was also used for the analysis of a series of pectins obtained by de-esterification of the DM93 pectin with tomato PME (Figure 6). It can be seen from the elution profiles for the control (solubilised at pH 8) that some chemical saponification occured (Figure 6; blank) resulting in a chemical modification of the pectic populations in contrast to

the blank solubilised at pH 4.5 (Figure 5). Furthermore, modifications by using tomato PME (Figure 6) lead to an increase of pectin populations eluting at high ionic strength: from 0 till 45% of the total peak area was eluted between 10-17min. The presence of this very broad peak indicated a non-homogenous pectin sample (Figure 6). In contrast to the *Aspergillus aculeatus* PME results, the peak corresponding to very high DM pectins (~ 1.5 min) was not degraded. The second pectic population (~ 4 min) was more degraded since it represents 60% of the total peak area in the blank and only 38% in the pectin Tom66. This indicated a preference for this population by tomato PME.

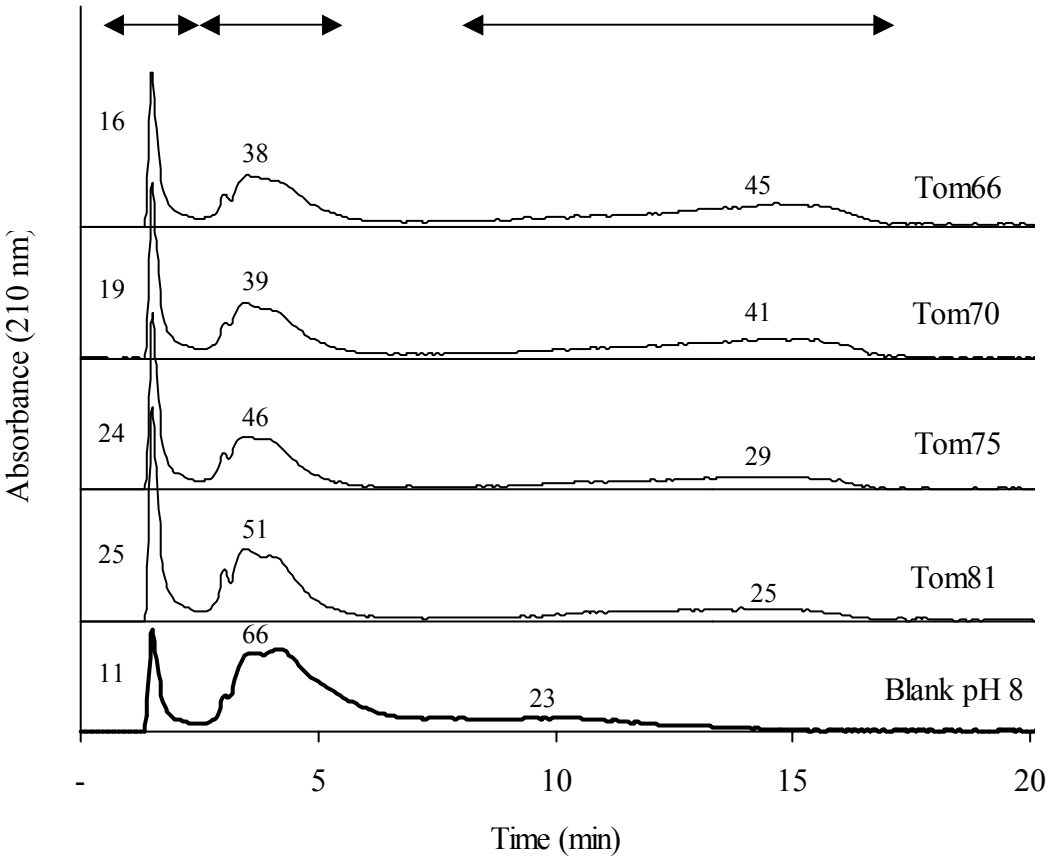

Figure 6: WAX elution profiles after background correction of de-methyl-esterified pectins with tomato PME with a DM of 66% (Tom66), 70% (Tom70), 75% (Tom75), 81% (Tom81) and pectin M93 (at pH 8.0) without enzyme (Blank pH 8.0). The arabic numbers indicate the peak area in percentage compared to the total peak area.

In the literature, it has been reported that plant PME cannot act on a fully methyl-esterified pectin and needs a minimum of 5% free carboxyl groups for degradation (Massiot, Perron, Baron & Drilleau, 1997; Solms & Deuel, 1955). This may explain why the HM pectin

population (~ 1.5 min) was not modified: its DM was too high to allow the action of tomato PME. However, the plant PME was able to de-esterify the second population to a higher extent than the fungal PME. This behavior can be explained by the fact that the enzyme is degrading pectin polymers in a single chain mechanism (Kohn et al., 1968).
Previous studies indicated that plant PME was able to degrade pectins until a DM around 40 % corresponding to its de-esterification limit (Massiot et al., 1997). Our results showed that some pectic populations were eluting at the same elution time as PGA pointing to a higher de-esterification level. Another explanation may be that pectins with large blocks of non-methyl-esterified galacturonic acid residues eluted similarly as PGA. Different elution profiles have been observed by Schols et al. (1989) when using citrus pectin esterase. These authors found more heterogenous pectic populations compared to our study and this difference may be explained by a different ratio enzyme-pectin used. With a high dose of enzyme several polymers can be de-esterify resulting in numerous but short de-esterified GalA blocks while a lower enzyme dose creates less but larger de-esterified GalA blocks. It is also possible that the differences in elution profiles are due to the absence of chemical saponification since Schols et al. (1989) de-esterified pectins with the plant PME at pH 7 instead of pH 8 used in this study.

Conclusions

Pectins were separated according to the total charge as it has been shown previously (Schols et al., 1989). Amidated pectins were also separated according to the same mechanism as methyl-esterified pectins. Amidated moieties were indeed recognised as non-charged GalA residues such as methyl-esters and they did not contribute to the binding of the pectins to the anion exchanger. Finally, pectins with random distribution of the methyl-esters seemed to behave differently on the anion exchange material compared to pectins with a blockwise distribution of the methyl-esters.

References

Ahmed A. E. R., Labavitch J. M. (1977). *Journal of Food Biochemistry*, *1*, 361-365.

Anger H., Dongowski G. (1984). Studies on the distribution pattern of free carboxyl groups in pectins by fractionation on deae-cellulose. *Die Nahrung.*, *28*, 199-206.

Barrett A. J. B., Northcote D. H. (1965). Apple fruit pectic substances. *Biochemical Journal*, 94, 617-627.

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

Christensen P. E. (1954). Methods of grading pectin in relation to the molecular weight (intrinsec viscosity) of pectin. *Food Research*, *19*, 163.

Daas P. J. H., Meyer-Hansen K., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research*, *318*, 135-145.

Darvill A. G., McNeill M., Albersheim P. (1978). Structure of plant cell walls: VIII. A new pectic polysaccharide. *Plant Physiology*, 62, 418-422.

De Vries J. A., Voragen A. G. J., Rombouts F. M., Pilnik W. (1981). Extraction and purification of pectins from alcohol insoluble solids from ripe and unripe apples. *Carbohydrate Polymers*, *1*, 117-127.

Guillotin S. E., Bakx E. J., Boulenguer P., Mazoyer J., Schols H. A., Voragen A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectin which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*, 391-398.

Heri W., Neukom H., Deuel H. (1961). Chromatographische fraktionierung von pektinstoffen an diathylaminoathyl-cellulose. *Helvetica Chimica Acta*, *XLIV*, (VII), 239.

Ishii S., Kiho K., Sugiyama S., Sugimoto H. (1979). Low-methoxyl pectin prepared by pectinesterase from *Aspergillus japonicus*. *Journal of Food Science*, *44*, 611-614.

Klavons J. A., Bennett R. B. (1986). Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. *Journal of Agricultural and Food Chemistry*, *34*, 597-599.

Kohn R., Furda I., Kopec Z. (1968). Distribution of free carboxyl groups in the pectin molecule after treatment with pectin esterase. *Collection of Czechoslovak Chemical Communications*, *33*, 264-269.

Kravtchenko T. P., Berth G., Voragen A. G. J., Pilnik W. (1992). Studies on the intermolecular distribution of industrial pectins by means of preparative size exclusion chromatography. *Carbohydrate Polymers*, *18*, 253-263.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992a). Analytical comparison of three industrial pectin preparations. *Carbohydrate Polymers*, *18*, 17-25.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992b). Studies on the intermolecular distribution of industrial pectins by means of preparative ion-exchange chromatography. *Carbohydrate Polymers*, *19*, 115-124.

Laurent M. A., Boulenguer P. (2003). Stabilization mechanism of acid dairy drinks (ADD) induced by pectin. *Food Hydrocolloids*, *17*, 445-454.

Lecacheux D., Brigand G. (1988). Preparative fractionation of natural polysaccharides by size exclusion chromatography. *Carbohydrate Polymers*, *8*, 119-130.

Lofgren C., Guillotin S., Evenbratt H., Schols H., Hermansson A.-M. (2005). Effects of calcium, pH and blockiness on kinetic rheological behavior and microstructure. *Biomacromolecules*, *6*, 646-652.

Massiot P., Perron V., Baron A., Drilleau J. F. (1997). Release of methanol and depolymeriztion of highly methyl esterified apple pectin with an endopolygalacturonase from *Aspergillus niger* and pectin methylesterases from *A. niger* or from Orange. *Lebensmittel Wissenschaft und Technologie*, *30*, 697-702.

May C. D. (1990). Industrial pectins: Sources, production and applications. *Carbohydrate Polymers*, *12*, 79-99.

McNeill M., Darvill A. G., Albersheim P. (1980). Structure of plant cell walls : X. Rhamnogalacturonan I A structurally complex polysaccharide in the walls of suspension-cultured sycamore cells. *Plant Physiology*, 66, 1128-1134.

Neukom H., Amado R., Pfister M. (1980). Neuere erkenntnisse auf dem gebiete der pektinstoffe. *Lebensm.wiss.u technology*, 13, 1-6.

O'Neill M. A., Ishii T., Albersheim P., Darvill A. G. (2004). Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review of Plant Biology*, *55*, (1), 109-139.

Owens H. S., Svenson H. A., Schultz T. H., (1933). In: *Natural Plant Hydrocolloids, Advances in Chemistry*; Washington DC, Ameridan Chemical Society, 10.

Pilnik W., Voragen A. G. J., (1991). In: *Food Enzymology;* London, Elsevier Applied Science, 303-306.

Ralet M. C., Bonnin E., Thibault J.-F. (2001). Chromatographic study of highly methoxylated lime pectins de-esterified by different pectin methyl-esterases. *Journal of Chromatography B*, *753*, 157-166.

Ralet M. C., Thibault J.-F. (2002). Interchain heterogeneity of enzymatically deesterified lime pectins. *Biomacromolecules*, *3*, 917-925.

Rolin C., (2002). Commercial pectin preparations. In: *Pectins and their Manipulation;* Seymour G. B., Knox J. P., Blackwell Publishing Ltd, 222-239.

Schols H. A., Reitsma J. C. E., Voragen A. G. J., Pilnik W. (1989). High-performance ion exchange chromatography of pectins. *Food Hydrocolloids.*, *3*, (2), 115-121.

Solms J., Deuel H. (1955). Uber den mechanismum der enzymatischen verseifung von pektinstoffen. *Helvetica Chimica Acta*, *38*, 321-329.

Thibault J.-F. (1979). Automatisation du dosage des substances pectiques par la méthode au méta-hydroxydiphenyl. *Lebensmittel Wissenschaft und Technologie*, *12*, 247-251.

Thibault J.-F., Rinaudo M. (1986). Chain association of pectic molecules during calcium-induced gelation. *Biopolymers*, *25*, 456-468.

Tollier M. T., Robin J. P. (1979). Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine. *ann. techno. agric.*, *28*, (1), 1.

Van Deventer-Schriemer W. H., Pilnik W. (1987). Studies on pectin degradation. *Acta Alimentaria*, *16*, 143.

Vincken J.-P., Schols H. A., Oomen R. J. F. J., McCann M. C., Ulvskov P., Voragen A. G. J., Visser R. G. F. (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology*, *132*, 1781-1789.

Voragen A. G. J., Pilnik W., Thibault J.-F., Axelos M. A. V., Renard C. M. G. C., (1995). Pectins. In: *Food polysaccharides and their applications*; Stephen A. M., New York: Marcel Dekker Inc., 287-339.

Wood P. J., Siddiqui I. R. (1971). Determination of methanol and its application to measurement of pectin ester content and pectin methyl-esterase activity. *Analytical Biochemistry*, *93*, 418-428.

Chapter 3

Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities.

Published as
S.E. Guillotin, E.J. Bakx, P. Boulenguer, J. Mazoyer, H.A. Schols and A. G. J. Voragen. Carbohydate polymers: 30, 391-398 (2005).

Abstract

Two commercially extracted pectins having different physical properties but similar chemical characteristics were fractionated into sub-populations by using ion exchange chromatography. Individual sub-populations were characterised by using established strategies (galacturonic acid and neutral sugar content, degree of methyl-esterification) including the use of enzymes (endo- and exo-polygalacturonases) as analytical tool. Some purified populations showed similar degree of methyl-esterification whereas they were eluting at different ionic strength. It was shown that these populations mainly differed in the number of galacturonic acid moieties in 'endo-polygalacturonase degradable blocks' and in the location of these blocks within the molecule. The size of the blocks present at the non-reducing end of the pectin was also different within the molecules. The separation of pectins on anion exchanger combined with the use of enzymes allowed us to differentiate between pectic sub-populations. Commercial pectins appeared to be a mixture of several polymers differing in total charge as well as in the distribution of the charges.

1. Introduction

Pectins are mainly present in the primary cell wall and in the middle lamella of plants. They constitute around 40 % (dry matter basis) of the cell wall of fruits and vegetables (Brett & Waldron, 1996). The nature of pectin depends on the origin, the growing and harvesting conditions of the crop and also on its localisation in the plant tissue and cell wall. Pectins are complex mixtures of polysaccharides composed of a galacturonic acid backbone (homogalacturonan or so-called smooth regions) of which variable proportions can be methyl-esterified. In addition, so-called hairy regions are present, constituted of alternative sequences of rhamnose and galacturonic acid (rhamnogalacturonan I) carrying variously sized neutral side chains (arabinans, arabinogalactans) attached to rhamnose moieties (Pilnik & Voragen, 1991; Voragen, Pilnik, Thibault, Axelos & Renard, 1995). Pectins are used as food ingredients mainly for their gelling properties, while also pharmaceutical properties as antidiarrhea, detoxicant, regulation and protection of gastrointestinal tract and anti-tumour activity have been mentioned (Voragen et al., 1995; Waldron & Selvendran, 1993). Different plant materials are used for the extraction of pectins (e.g. citrus peel, apples pomace and sugar beet pulp) and differences in functional properties are observed according to the process and origin of the raw material. It is known that gelling properties of commercial pectins strongly depend on the degree of methyl-esterification of the galacturonic acid residues (Voragen et al., 1995). Nevertheless, various pectins with similar chemical characteristics (galacturonic acid (GalA) and neutral sugar (NS) content, degree of methyl-esterification (DM)) may behave differently in gel formation.

In addition to the common chemical characterisation of pectins (determination of the GalA content and DM), new parameters to distinguish pectins were introduced (Daas, Alebeek, Voragen & Schols, 1999; Daas, Arisz, H.A., De Ruiter & Voragen, 1998; Daas, Meyer-Hansen, Schols, De Ruiter & Voragen, 1999; Daas, Voragen & Schols, 2000, 2001; Korner, Limberg, Mikkelsen & Roepstorff, 1998; Limberg et al., 2000). Daas et al. used enzymatic degradation of the pectins with an endo-polygalacturonase of *Kluyveromyces fragilis* and analysed the partially methylated oligogalacturonides released. From these data they defined the degree of blockiness (DB) of pectins represented by the amount of non methyl-esterified mono-, di- and trigalacturonic acid released by the enzyme relative to the total amount of non-methylesterified galacturonide residues present in the pectin. The higher the DB of pectins having a similar DM, the more blockwise the distribution of the methyl-esters in the pectin.

Pectins having similar DM and DB values, may still differ in the size of the blocks. This difference can be characterised by a second parameter: the proportion of mono-, di- and trigalacturonic acid in the endo-PG digests. Long degradable blocks will lead to the release of high amounts of di- and trigalacturonic acid compared to monogalacturonic acid upon enzymatic digestion. The third parameter described by Daas et al. is the ratio of the total peak area of oligomers with methyl-esters to the total of peak areas of oligomers without methyl-esters (Me^+/Me^- ratio). This is an indication of the location of the degradable blocks within the backbone: the higher this ratio, the more clustered are the degradable blocks distributed over the pectin molecule (Daas, Alebeek et al., 1999; Daas et al., 1998; Daas, Meyer-Hansen et al., 1999; Daas et al., 2000).

Chromatography performed on an anion exchange column (Schols, Reitsma, Voragen & Pilnik, 1989) or size exclusion column (Kravtchenko, Berth, Voragen & Pilnik, 1992) showed that commercial pectins were not composed of one single pectic population but they are constituted of various populations with different features. Anger and Dongowski (1984), Schols et al. (1989) and Kravtchenko, Voragen, and Pilnik (1992) suggested that elution on an anion exchange column may vary according to the degree of methyl-esterification, but as well as to the distribution of the charges. More recently, Ralet and Thibault (2002) studied the effect of charge distribution on the behavior on an anion exchanger of pectins demethylated by plant PME or fungus PME using conductometric measurements. In their study they could not show any influence of the charge distribution on the elution on an anion exchanger.

Until now, the approach of Daas, Alebeek et al. (1999), Daas et al. (1998), Daas, Meyer-Hansen et al. (1999), Daas et al. (2000, 2001) using the DB, mono-, di- and trigalacturonic proportions and the Me^+/Me^- ratio of a pectin preparation “as is” has not been extended with fractionation of the pectin into sub-populations and characterisation of these sub-populations. This approach would enable the estimation of the intramolecular distribution (distribution of methyl-esters within one pectic molecule) as well as the intermolecular distribution of methyl-esters (distribution of methyl-esters over several pectic molecules). These distributions are expected to be related to the gelling behaviour in the presence of calcium. In this research, two pectins having different calcium reactivity and extracted from the same raw material with similar chemical characteristics are studied using these state-of-the-art approaches and tools.

2. Experimental

2.1. Samples

The samples were kindly provided by Degussa Texturant Systems (Baupte, France). Pectins A and B were selected for having nearly the same degree of methyl-esterification (DM of 74% and 72%, respectively), galacturonic acid content (GalA of 82% w/w and 74 % w/w, respectively) and intrinsic viscosity but different calcium sensitivity (Laurent & Boulenguer, 2003). The intrinsic viscosity and the calcium sensitivity have been published already (Laurent & Boulenguer, 2003). Pectin A, also called calcium sensitive (CS) pectin and pectin B, also called Non Calcium Sensitive (NCS) have an intrinsic viscosity of 723 and 739 ml/g and a calcium sensitivity of 297 $mPa.s^{-1}$ and 39 $mPa.s^{-1}$, respectively (Laurent & Boulenguer, 2003).

2.2. Size exclusion chromatography of pectins

High-performance size exclusion chromatography (HPSEC) was performed with three Tosoh Biosciences TSK gel columns (G 4000, 3000, 2500 PWXL, each 300 × 7.5 mm) in series and in combination with a PWXL guard column (Tosoh Biosciences; 40 × 6 mm). Elution was performed at 30° C with 0.2 M sodium nitrate at 0.8 ml/min. The eluate was monitored using a Shodex SE-61 refractive index detector. Twenty µl of pectin (5 mg/ml) was injected.

2.3. Preparative chromatography of commercial pectins

An Akta explorer system was used for separation of pectins on a preparative scale. Pectin (0.5 g) was dissolved in 100 ml of 0.03 M of sodium phosphate buffer. Elution was performed on a Source-Q column (115 × 60 mm; Amersham Biosciences) using 'Millipore' water during 4 column volumes (CV) followed by a linear gradient in steps: 0-0.12 M of sodium phosphate buffer (pH 6) in 13 CV at 60 ml/min; 0.12-0.42 M of sodium phosphate buffer (pH 6) in 44 CV; 0.42-0.6 M sodium phosphate (pH 6) in 2 CV and finally 8.5 CV of 0.6 M sodium phosphate pH 6. The column was washed with 1 M sodium hydroxide for 5 CV. Detection was accomplished with an UV detector set at 215 nm.

The fractions (250 ml) were pooled and ultrafiltrated with a Pellicon 10 kDa membrane (size of 50 cm^2) till a conductivity of < 10 µS. After ultrafiltration, the fractions were freeze-dried.

Then the different pools were resuspended and dialysed with dialysis tubing (cut of 12-14 kDa for proteins) against 'Millipore water' to remove last traces of salts prior to freeze-drying.

2.4. Uronic acid and neutral sugar content

Pectins (60 μg/ml) were boiled (1h), cooled and then saponified with sodium hydroxide (40mM). The uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl method (Ahmed & Labavitch, 1977; Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979). Total neutral sugars were estimated with the automated orcinol method (Tollier & Robin, 1979), using galactose as a standard. Populations A5 and B5 were not soluble, so, a pre-hydrolysis step with sulfuric acid (72% in w/w) was performed on these samples prior to the colour reaction.

2.5. Neutral sugar content

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984) using inositol as an internal standard. The samples were treated with 72% (w/w) H_2SO_4 (1h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C and the constituent sugars released were analysed as their alditol acetates.

2.6. Methyl-ester content

The methyl-ester content was determined by GC headspace analysis of the free methanol released after alkaline de-esterification of pectins (Huisman, Oosterveld & Schols, 2004).

2.7. Degree of blockiness

The degree of blockiness has been determined as described previously (Daas, Meyer-Hansen et al., 1999; Daas et al., 2000). Samples (5 mg/ml) were diluted in sodium acetate 50 mM pH5 and incubated with an overdose of endo-polygalacturonase of *Kluyveromyces fragilis* (0.04 units/ml) for 24 hours. The specific activity of this enzyme for PGA was 128 U/mg. Pectin digests were prepared by incubation of pectic solution with endo-polygalacturonase (0.04 units/ml) for 24 hours. As a result of the extended endo-polygalacturonase incubation employed, end-products were observed as was demonstrated by the use of an excess of

enzymes and longer incubation times. EDTA (0.024 mM) was added to solubilise poorly soluble pectins (populations A5 and B5) prior to enzymatic digestion. Oligomers released were analysed by using HPAEC (80 µl injection) equipped with a Dionex CarboPac PA1 anion exchange column (250 × 4 mm) and a CarboPac PA1 precolumn (50 × 4 mm). Elution was performed with sodium acetate at pH 5 from 0.05 to 0.7 M in 65 min with a flow of 0.5 ml/min. The gradient was hold at 0.7M sodium acetate for 5 min. The PAD detector (Dionex) was equipped with a gold working electrode and an Ag/AgCl reference electrode. Detection of the oligomers took place after post column addition of sodium hydroxide (1 M; 0.5 ml/min). The degree of blockiness (DB) is the amount of mono- di- and trigalacturonic acid released by the endo-polygalacturonase related to the amount of free GalA present in the sample (Figure 1).

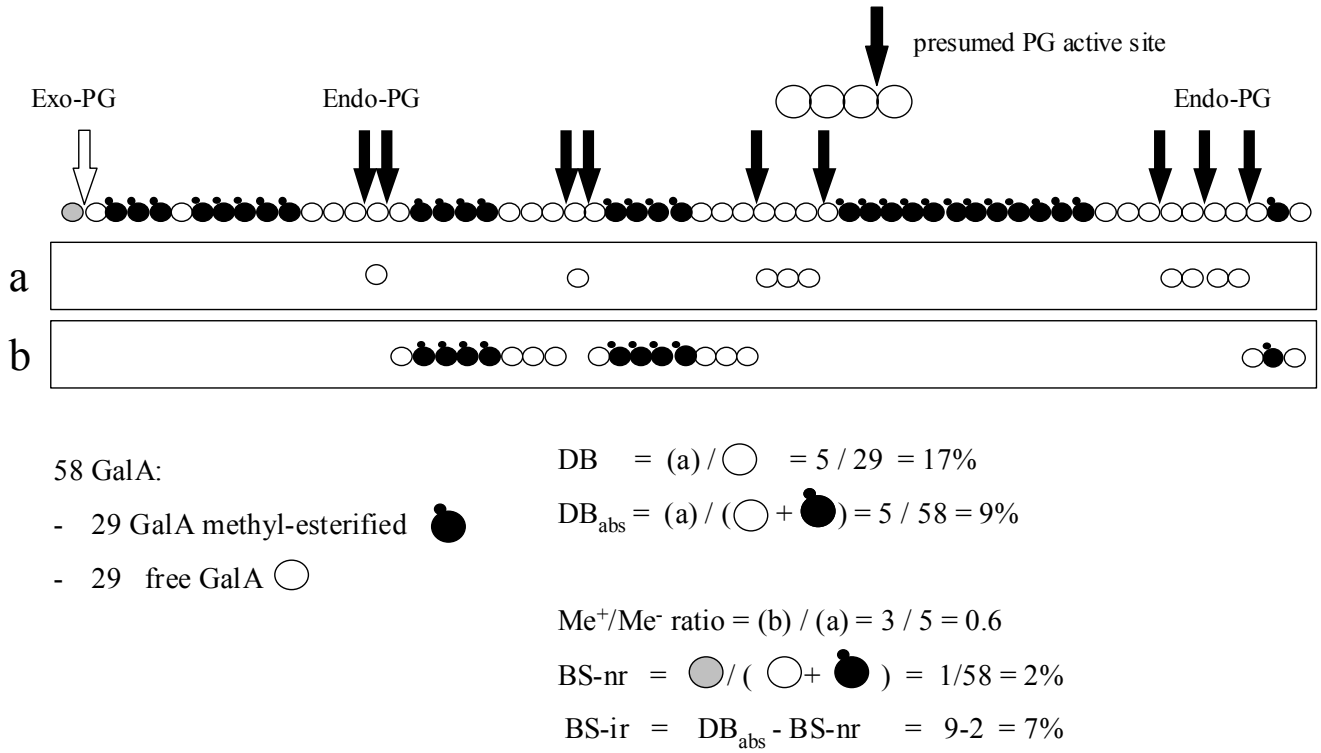

Figure 1: Schematic representation of enzymatic digestion with endo-PG from *Kluyveromyces fragilis* (endo-PG*)* and exo-PG from *Aspergillus tubingensis* (exo-PG) on a 50% DM pectin. Description of the parameters: DB, DB$_{abs}$, Me$^+$/Me$^-$ ratio, BS-nr, BS-ir. It is assumed that endo-PG needs 4 adjacent non-esterified GalA residues to act (Daas, Meyer-Hansen et al., 1999). White and black arrows indicate the action of the endo-PG and exo-PG respectively. Oligomers released by endo-PG and small enough to be analysed on HPAEC pH5 are indicated as (a) for the non-methylesterified ones and (b) for the methyl-esterified ones. GalA molecules released by exo-PG are indicated in ●.

The absolute degree of blockiness (DB_{abs}) is the amount of mono- di- and trigalacturonic acid released by the endo-polygalacturonase related to the total amount of GalA (free and methyl-esterified GalA) present in the sample (Figure 1).

2.8. Free GalA blocks at the non-reducing end

Samples (5mg/ml) were diluted in sodium acetate 50 mM and incubated with exo-polygalacturonase of *Aspergillus tubingensis* (Kester, Someren, Muller & Visser, 1996). The specific activity of this enzyme for PGA was 118U/mg. Pectin digests were prepared by incubation of pectic solution with exo-polygalacturonase (0.04 units/ml) for 24 hours. MonoGalA from the exo-polygalacturonase digests samples was analysed on HPAEC at pH12 equipped with a Dionex Carbopac PA1 column (250 × 4 mm) and a CarboPac PA-1 precolumn (50 × 4 mm). Sample (50 µl of 5 mg/ml pectin digest) was injected on the column and elution started with a pre-equilibration step of 15 min with 0.1 M NaAcetate in 0.1 M NaOH (1 ml/min) followed by a linear gradient of 1 M NaAcetate in 0.1 M NaOH (0.01 M-1 M during 60 min) and a washing step of 5 min with 1 M NaAcetate in 0.1 M NaOH. Oligomers were detected with a PAD-detector (Dionex) equipped with a gold working electrode and an Ag/AgCl reference electrode. During each series, the PAD response area of a standard amount of monoGalA (0.2 mg/ml) was determined. The amount of free GalA present at the non reducing end related to the amount of total GalA in the sample is determined and defined as the so-called Block Size at the Non Reducing end; BS-nr (Figure 1). The amount of GalA present interior and/or at the reducing end of the sample is determined as well (so-called Block Size Interior and/or at the Reducing end; BS-ir, Figure 1).

3. Results and discussion

3.1. Fractionation of commercial pectin preparations in sub-fractions on preparative anion exchange chromatography

Two commercial HM pectins (pectins A and B) originating from the same citrus variety showed totally different gelling properties in presence of calcium. The chemical characteristics (GalA, NS, DM; Table I) of these pectins did not explain the different gelling behavior since they were similar. Schols et al. (1989) were able to separate pectic populations present in commercial pectins with different degree of methyl-esterification (DM) according

to their charges, using an HPLC system equipped with an anion exchange column (MA7P column). The charge level and charge distribution seemed to have an influence on the elution behaviour of the pectins. Since the column used by Schols et al. was not commercially available, a column giving similar results was used (Propac^{TM} WAX-10 column, Dionex). Pectins A and B showed totally different elution profiles (results not shown). To enable a detailed characterisation of the individual populations and to understand the different physical behavior, pectins A and B were fractionated on preparative scale using a Source-Q column (Figure 2).

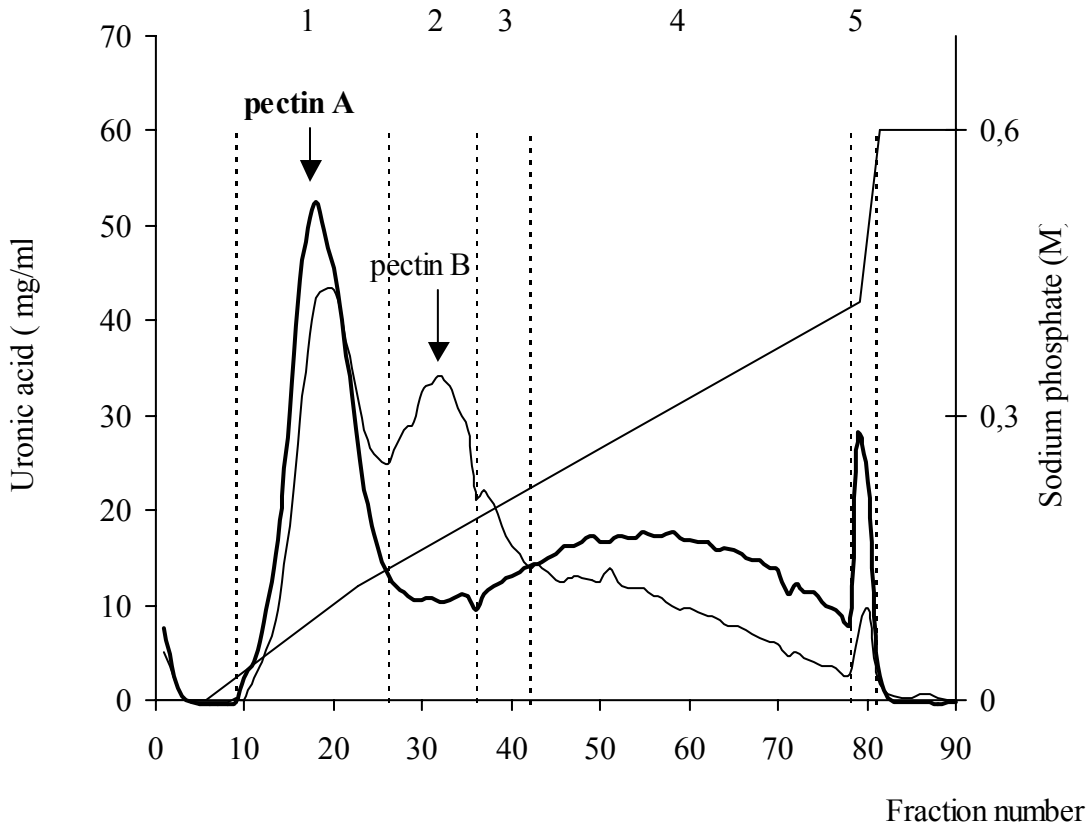

Figure 2: Preparative anion-exchange chromatography of pectins A and B on Source-Q column. The elution profiles were obtained after determination of the uronic acid content in each fraction. The fractions (250 ml) were pooled as indicated.

Elution profiles obtained with the Source-Q column (Figure 2) were similar to those obtained with the analytical WAX column: pectins A and B showed several pectic populations in different relative amounts. Next to a detailed characterisation of the individual populations also the mechanism for the different elution behaviour of the various pectic sub-fractions was

Table I : GalA content, yield, degree of methyl-esterification (DM) and degree of blockiness (DB) and methyl to non-methyl-esterified peak area ratio of pectins A, B and corresponding fractions obtained by chromatography over Source-Q.

samples	GalA (w/w%)	Yield (%)[a]	NS (w/w%)	DM (%)	DB (%)[b]	DB_{abs} (%)[c]	Methyl- to non-methyl-esterified area ratio	BS-nr (%)[d]	BS-ir (%)[e]
A	82		7	74	16	4.2	0.1	1	3.2
A1	82	39	1	86	3	0.4	1.5	0.1	0.3
A2	59	6	4	85	15	2.2	0.7	0.6	1.7
A3	62	6	5	86	18	2.5	0.4	0.4	2.1
A4	75	44	4	69	15	4.6	0.2	1.2	3.4
A5	57	5	3	44	40	22.4	0.0	1.7	20.7
B	74		12	72	5	1.4	0.9	0.8	0.6
B1	70	32	2	92	4	0.3	2.8	0.2	0.2
B2	69	26	2	78	6	1.3	1.9	0.4	0.9
B3	75	11	4	59	3	1.2	0.2	0.8	0.41
B4	65	29	5	64	34	12.2	0.1	5	7.2
B5	32	2	4	40	35	21	0.1	nd	nd

nd: not determined

[a] GalA yield is expressed as percentage of all GalA residues recovered

[b] amount of mono- di- and triGalA released by the enzyme related to the amount of free GalA present in the sample

[c] amount of mono- di- and triGalA released by the enzyme related to the total amount of GalA: [(100-DM) * DB/100]

[d] free GalA present at the non reducing end also called Block Size at the Non Reducing end (BS-nr) related to the total amount of GalA in the sample

[e] free GalA present interior and/or on the reducing end also called Block Size Interior and/or on the Reducing end (BS-ir) related to the total amount of GalA

subject for further studies. So fractions were pooled, ultra-filtrated before freeze-drying and further analysed.

3.2. Chemical characterisation of the different populations obtained after preparative Source-Q chromatography

3.2.1. Galacturonic acid and neutral sugar content in pectic populations

The recovery of pectin was measured by comparison of the GalA content of the injected sample and the GalA content measured in all fractions. Pectins A and B were recovered after chromatography for 76% and 79%, respectively. These values are not uncommon in this scale of chromatography (Kravtchenko, Voragen et al., 1992). Fractions eluted from the column were analysed for GalA content, DM and DB. The length and distribution of the blocks were also studied (Figure 1). It is shown in Table I that the GalA content was quite high for populations 1-4 from both pectins A and B and lower for the populations eluted with a higher ionic strength (populations A5 and B5). This phenomenon was observed previously by Kravtchenko, Berth et al. (1992). The neutral sugar content was low for all the populations (from 1 to 5%, w/w). The non-carbohydrate material may be due to insufficient removal of salts by ultrafiltration.

3.2.2. Degree of methyl-esterification in the various pectic populations

As charge and charge density are the most important parameters that influence the elution of pectic polysaccharides from an anion exchange column (Schols et al., 1989), the DM of all the pectin pools was determined. In general, pectic molecules with a lower DM were bound more strongly to the column and needed thus higher salt concentrations to be eluted (Table I). This is in agreement with the findings of Kravtchenko, Berth et al. (1992) for lemon pectins eluted from a DEAE-Sepharose column. However, some populations with similar DM were found to elute at different buffer concentrations: sub-population A1, A2 and A3 (all DM 86) eluted at 0.1, 0.17 and 0.21 M buffer respectively. On the other hand, populations A3 and B3 presented different DM values whereas they eluted at the same ionic strength. These observations may be explained by a different distribution of the methyl-esters over the pectin backbone. To check this hypothesis the degree of blockiness, reflecting the distribution of methyl-esters over the pectic backbone, was determined. Some results are in contrast with

Kravtchenko, Berth et al. (1992) since the latest pectins eluted (A5 and B5) presented a low DM (44% and 40% DM, respectively).

3.2.3. Degree of blockiness of the pectic populations

All pectic populations were digested with polygalacturonase of *Kluyveromyces fragilis* (PG*kf*) and degradation products were analyzed and quantified using HPAEC pH 5. All oligomers observed in the elution pattern have been previously identified using Maldi-TOF MS (Daas et al., 1998). From these results, two different parameters were determined: the DB and the Me^+/Me^- ratio (Daas, Meyer-Hansen et al., 1999; Daas et al., 2000) (Table I). A high DB value is indicative for a blockwise distribution of non-esterified galacturonic acid residues in a pectin. The Me^+/Me^- ratio is indicative for the distribution of the non-esterified GalA 'blocks' over the pectin backbone (Daas et al., 2000). The higher this ratio, the closer the non-esterified GalA 'blocks' are. The mother pectin B presented a more random distribution of the methyl-esters than parental pectin A since the DB is 5% for pectin B and 16% for pectin A (Table I). These values fit in the range mentioned by Daas, Meyer-Hansen et al. (1999): DB of 1% for a random DM70 pectin (R70) and DB of 11% for a blockwise DM70 (B71).

To check whether the populations of pectins A and B had different charge distributions, the DB of each sub-fraction was analyzed. For similar DM pectins (populations A1- A3), the DB value is increasing for populations eluting at higher ionic strength (DB of 3% for pectin A1, 15% for pectin A2 and 18% pectin A3). As expected on forehand, the more blockwise the distribution of free GalA residues within the pectin (higher DB), the later the pectin eluted. The DB gives information about the presence of blocks (3 to 18% of all non-esterified GalA residues are grouped for pectin A1-A3). It is obvious that the DB is not enough to explain the elution behavior of the sub-populations on the anion exchanger. For example, populations A3 and A4 are both blockwise pectins but the DM is different so the proportion of blocks is different. Taking this into account, we introduced the DB_{abs} (Figure 1). This parameter gives information about the absolute number of blocks in the pectin samples without correction of the DM (Figure 1). It is clear that blocks of free GalA were influencing the elution behavior of the pectins: the more blocks of non-methyl-esterified GalA in the pectic sample, the later the elution is (Table I). The DB_{abs} was increasing from 0.4% for A1 to 22% for A5 and from 0.3% for B1 to 21% for B5. Only fraction B2 was slightly deviating from this rule. This fraction B2 contained a few more blocks or larger ones than pectin B3, while these blocks

were closer to each other compared to pectin B3 (Me^+/Me^- ratio was, respectively, 1.9 and 0.2). Anger and Dongowski (1984), Schols et al. (1989) and Kravchentko, Voragen et al. (1992) explained the elution behavior by the charge distribution. Our results confirmed this hypothesis although our findings differed from the results published recently by Ralet and Thibault (2002) using a DEAE-Sepharose CL-6B column for chromatography of pectins.
It has also been noticed above, that pectins A3 and B3 eluted at the same ionic strength whereas their DM were different (86% and 59%, respectively). The DB_{abs} of population A3 (2.5%; Table I) was higher compared to DB_{abs} of population B3 (1.2%). Also the Me^+/Me^- ratio of fraction A3 was twice as high than that for fraction B3 (0.4% and 0.2%, respectively). So less degradable blocks were present in pectin B3, but more distant from each others compared to the pectin A3. This may explain their similar binding on the anion exchanger. Obviously, the co-elution of a 86% DM pectin having some blocks of GalA residues with a random 59% DM pectin complicated the interpretation of anion exchange patterns, but the enzymatic degradation of these populations showed us that these pectins were different concerning the amount and distribution of free GalA blocks. Another surprising finding was the co-elution of populations A2 and B2 with similar DM but different DB_{abs} (2.2% and 1.3%, respectively) and Me^+/Me^- ratio (0.7 and 1.9, respectively). Population B2 contained less 'endo-PG degradable' blocks more clustered compared to population A2. These data revealed that the anion exchange column does not make any distinction between the 'random' pectin B2 with some clustered but rather short GalA blocks and the blockwise pectin A2 with only few, more distant blocks. Our findings clearly showed that the column was not able to distinguish between all different pectin populations present, but the enzymatic degradation of the pectins showed that this populations presented different endo-PG degradable blocks.

3.2.4. Does the molecular size distribution influence the behaviour of the pectic populations in anion exchange chromatography?

To establish whether the molecular size of the various populations could explain their behaviour on the anion exchange column, each pectic population was analysed by high performance size exclusion chromatography (HPSEC). It can be seen that the molecular size was slightly higher for pectins eluting at high ionic strength (Figure 3) except for population A5. Since HPSEC elution profiles from most of the pectic populations showed rather similar molecular distribution in the range of 100-43 kDa (18-24 min; Figure 3), it could be

concluded that the size of the pectic polymers from the populations does not explain the different elution behaviour of the sub-fractions on the Source-Q column.

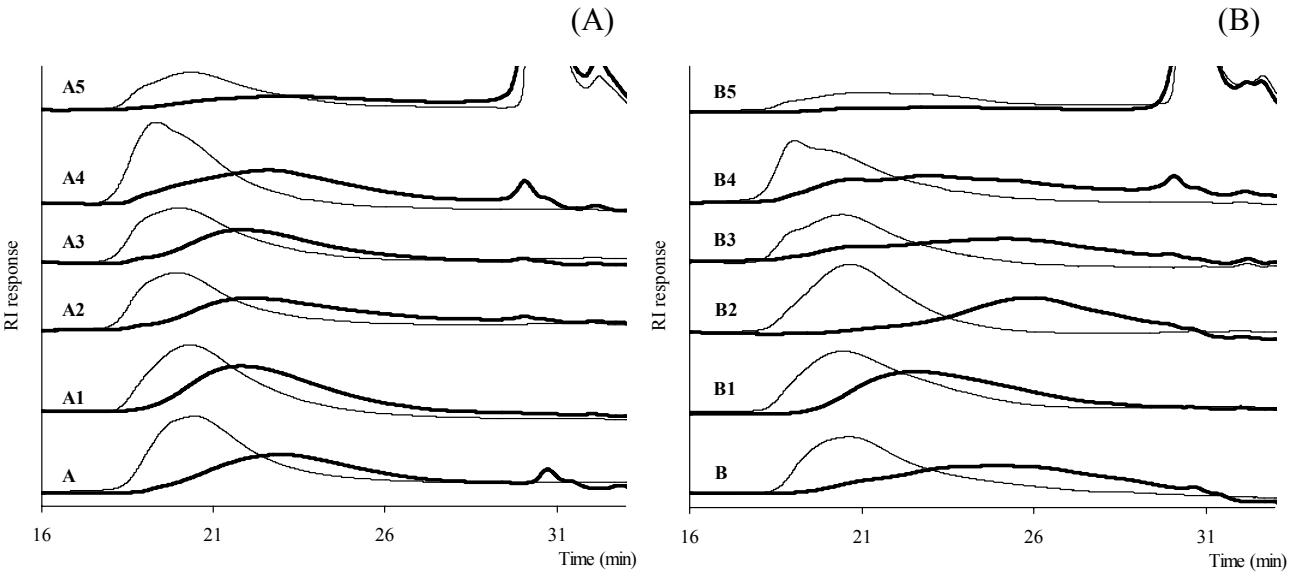

Figure 3: HPSEC elution profiles of the different populations obtained after preparative anion-exchange chromatography of pectins A (Figure A) and B (Figure B) on SourceQ column before (thin lines) and after degradation of pectins with endo-polygalaturonase from *Kluyveromyces fragilis* degradation (thick lines).

All pectin endo-PG digests were analysed by HPSEC as well. Next to the amount of mono-, di- and trigalacturonic acid released by the endo-PG and taken into account in the DB parameter, larger fragments are released which also give information on the distribution of methyl-esterified carboxyl groups over the galacturonan backbone. In general, the shift in Mw after PG digestion was more pronounced for pectin B populations than for pectin A populations (Figure 3). This Mw shift seems to be independent from the amount of small oligomers released (29-32 min) and these fragments (64-2 kDa) are not included in the calculation of any parameters described so far. Combining the results obtained from the DB calculation described above for pectins A2 and B2, and the HPSEC endo-PG digests profiles, it was concluded that these pectins were indeed totally different. Only a part of the pectin A2 molecule was well degradable by the endo-PG and this part was constituted of free GalA clusters. The other part of the pectin A2 was hardly degraded by the PG, which is subject for further research. For population B2, it was concluded that compared to population A2, smaller amounts of endo-PG degradable blocks were present, and that these blocks were more

randomly distributed over the pectic backbone. This explained the large decrease in Mw of pectin B2 compared to pectin A2. The same phenomenon was observed for populations A3 and B3.

3.2.5. GalA blocks present at the non reducing end, interior and/or at the reducing end

The presence of endo-PG degradable blocks at the extremities of the molecule may also lead to a less pronounced decrease in Mw for pectin A polymers compared to those of pectin B. To obtain more information about the localisation of free GalA blocks in the galacturonan backbone, an exo-polygalacturonase (exo-PG) was used to degrade the pectins. Since this enzyme is known to release only non-methyl-esterified GalA from the non-reducing end of pectins (Benen, Vincken & Alebeek, 2002) and needs two non-methyl-esterified GalA to act (only one free GalA at the non-reducing end is not a substrate for the exo-PG) (Korner, Limberg, Christensen, Mikkelsen & Roepstorff, 1999; Limberg et al., 2000), it is possible to determine whether pectins contain different block sizes of un-esterified GalA at the non-reducing end. Exo-PG digests were analysed with HPAEC at pH 12. Only monogalacturonic acid is released by the enzyme during pectin digestion. The amount of GalA released by the exo-PG, related to the total amount of GalA present on the pectin (Block Sequence on the Non Reducing end: BS-nr), was 1% for pectin A and 0.8% for pectin B. So pectin A was slightly more degraded with exo-PG (Table I). This indicated the presence of larger blocks of non-methyl-esterified GalA on the non-reducing end for pectin A. These results can be compared with previous results of Limberg et al. (2000) where the authors also analysed the exo-PG digests of a pectin with similar chemical characteristics as pectin A (blockwise pectin with a DM of 76, reference P76 in the publication). Based on their published values, we calculated that they found a BS-nr of 1.4 % which is in the same range of the BS-nr of 1% found in our study for pectin A. The BS-nr increased for populations eluting at increasing ionic strength except for populations A3 (BS-nr of 0.4% compared to 0.6% for population A2). In general, we can assume that the longer the block of free GalA on the non-reducing end of pectins, the later is the elution. The DB_{abs} is giving information on the block recurrence over the galacturonan backbone. The exo-PG is giving information about the GalA blocks present at the non-reducing end. Therefore, it is possible to determine the amount of blocks in the interior and/or in the reducing end of the galacturonan backbone by determining the BS-ir (Block Sequence Inside and on the Reducing end) parameter. This parameter was calculated

by substracting the DB_{abs} with the BS-nr (Block Sequence on the Non-Reducing end). From the data presented in Table I, we deduced that the higher the amount of GalA in block sequences located in the interior and/or on the reducing end, the stronger was the binding of the pectin on the anion exchanger. The BS-ir was 0.3% for population A1 and increased up to 20.7% for pectin A5. Population B3 was deviating from this rule. These results may explain the differences in elution behaviour of the pectic populations. Pectins were shown to be different from each other by different localisation of the free GalA blocks: some pectins presented more blocks on the non-reducing end, others on the reducing end and/or inside the pectic backbone.

Conclusions

Commercial pectins showed to be a mixture of different populations which can be separated on a preparative Source-Q column. This lead to differentiations between pectins with similar chemical features but different gelling behaviour. Separation of the pectins was depending on the DM as observed previously (Kravtchenko, Voragen et al., 1992; Schols et al., 1989) but the degree of blockiness (DB_{abs}) influenced the elution behaviour as well which is in agreement with previous suggestions (Schols et al., 1989). The position of the non methyl-esterified GalA blocks is also varying in the populations purified from the parental commercial pectin. Nevertheless, it is important to notice that some populations with similar DM and different distribution of the methyl-esters eluted at the same ionic strength, which made it difficult to interpret anion exchange elution patterns. The parameters described by Daas, Meyer-Hansen et al., (1999) and Daas et al. (2000) to characterise pectins in terms of size and type of distribution of free GalA blocks over the galacturonan backbone is not fully adequate. The parameters described in this study (DB_{abs}, BS-nr and BS-ir) and elaborated on the method of Daas, Meyer-Hansen et al., (1999) and Daas et al. (2000) and Limberg et al. (2000), provided further valuable information on the fine structure of more homogeneous pectic populations and on the behaviour of these pectins on an anion exchanger.
The combination of HPAEC and enzymatic digestion allowed us to visualise and characterise the different pectic polymers present in commercial pectins.

Acknowledgments: the authors would like to thank Dr Jac Benen who kindly provided the exo-polygalacturonase used in this study.

References

Ahmed A. E. R., Labavitch J. M. (1977). *Journal of Food Biochemistry*, *1*, 361-365.

Anger H., Dongowski G. (1984). *Die Nahrung.*, *28*, 199-206.

Benen J. A. E., Vincken J.-P., Alebeek G.-J. V., (2002). Microbial pectinases. In: *Pectins and their Manipulation*; Seymour G. A., Tucker G. B. , Blackwell Publishing Ltd., 174-215.

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

Brett C., Waldron K., (1996). Physiology and biochemistry of plant cell walls. Cambridge.

Daas P. J. H., Alebeek G. J. W. M. v., Voragen A. G. J., Schols H. A., (1999). Determination of the distribution of non-esterified glacturonic acid in pectin with endo-polygalacturonase. In: *Gums and Stabilisers for the food industry*; Williams P. A., Phillips G. O., Wrexham: The Royal Society of Chemistry, 3-18.

Daas P. J. H., Arisz P. W., H.A. S., De Ruiter G. A., Voragen A. G. J. (1998). Analysis of partially methyl-esterified galacturonic acid oligomers by high-performance anion-exchange chromatography and matrix-assisted laser desorption/ionization time-of flight spectronomy. *Analytical Biochemistry*, *257*, 195-202.

Daas P. J. H., Meyer-Hansen K., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research*, *318*, 135-145.

Daas P. J. H., Voragen A. G. J., Schols H. A. (2000). Characterisation of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research*, *326*, 120-129.

Daas P. J. H., Voragen A. G. J., Schols H. A. (2001). Study of the methyl ester distribution in pectin with endo-polygalacturonase and high performance size exclusion chromatography. *Biopolymers*, *58*, 195-203.

Englyst H. N., Cummings J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst*, *109*, 937-942.

Huisman M. M. H., Oosterveld A., Schols H. A. (2004). New method for fast determination of the degree of methylation of pectins by headspace GC. *Food Hydrocolloids.*, *18*, (4), 665-668.

Kester H. C. M., Someren M. A. K.-V., Muller Y., Visser J. (1996). Primary structure and characterisation of an exopolygalacturonase from *Aspergillus tubengensis*. *Euopean Journal of Biochemistry*, *240*, 738-746.

Korner R., Limberg G., Mikkelsen J. D., Roepstorff P. (1998). Characterization of enzymatic pectin digests by matrix-assisted Laser Desorption/Ionisation Mass Spectrometry. *Journal of Mass Spectrometry*, *33*, 836-842.

Korner R., Limberg G., Christensen T. M. I. E., Mikkelsen J. D., Roepstorff P. (1999). Sequencing of partially methyl-esterified oligogalacturonases by tandem mass spectrometry and its use to determine pectinase specificities. *Analytical Chemistry*, *71*, 1421-1427.

Kravtchenko T. P., Berth G., Voragen A. G. J., Pilnik W. (1992). Studies on the intermolecular distribution of industrial pectins by means of preparative size exclusion chromatography. *Carbohydrate polymers*, *18*, 253-263.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992). Studies on the intermolecular distribution of industrial pectins by means of preparative ion-exchange chromatography. *Carbohydrate polymers*, *19*, 115-124.

Laurent M. A., Boulenguer P. (2003). Stabilization mechanism of acid dairy drinks (ADD) induced by pectin. *Food hydrocolloids*, *17*, 445-454.

Limberg G., Korner R., Buchholt H. C., Christensen T. M. I. E., Roepstorff P., Mikkelsen D. J. (2000). Quantification of the amount of galacturonic acid residues in blocksequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger*. C*arbohydrate research*, *327*, 321-332.

Pilnik W., Voragen A. G. J., (1991). In: *Food Enzymology*; Fox P. F., London, Elsevier Applied Science, 303-306.

Ralet M. C., Thibault J.-F. (2002). Interchain heterogeneity of enzymatically deesterified lime pectins. *Biomacromolecules*, *3*, 917-925.

Schols H. A., Reitsma J. C. E., Voragen A. G. J., Pilnik W. (1989). High-performance ion exchange chromatography of pectins. *Food Hydrocolloids.*, *3*, (2), 115-121.

Thibault J.-F. (1979). Automatisation du dosage des substances pectiques par la methode au meta-hydroxydiphenyl. *Lebensm. -Wiss. u. -Technol.*, *12*, 247-251.

Tollier M. T., Robin J. P. (1979). *ann. techno. agric.*, *28*, (1), 1.

Voragen A. G. J., Pilnik W., Thibault J.-F., Axelos M. A. V., Renard C. M. G. C., (1995). Pectins. In: *Food polysaccharides and their applications*; Stephen A. M., New York, Marcel Dekker Inc., 287-339.

Waldron K. W., Selvendran R. R., (1993). In: *Food and cancer prevention, chemical and biological aspects*; Waldron K. W., Johnson I. T., Fenwick G. R., The Royal Society of Chemistry, 307-326.

Chapter 4

Determination of the degree of substitution, degree of amidation and degree of blockiness of commercial pectins by using capillary electophoresis.

To be submitted in Food Hydrocolloids as:
S.E. Guillotin, E.J. Bakx, P. Boulenguer, H.A. Schols and A. G. J. Voragen

Abstract

It is more and more realized that pectins are complex mixtures of many different molecules and research is directed towards the fractionation and characterization of these pectic sub-populations. Since fractionation of pectins generally results in only low amounts of purified material, rapid methods using low amounts of samples are required. In this study, capillary electrophoresis was chosen because only tiny amounts of sample are needed for the analysis. A new CE protocol was developed to determine the degree of amidation, the degree of methyl-esterification (DM) and consequently the degree of substitution (DS) of pectins by analyzing the pectins before and after removal of the methyl-esters. The CE results were compared with the results obtained by titration and FTIR spectroscopy methods. The CE method was found to be rather reliable with small standard deviations for the DS and DAm. The CE method had the advantage of being rapid due to the limited sample preparation and automation of the analysis. In addition, CE was used successfully to determine the degree of blockiness of the free GalA residues over the pectic backbone.

1. Introduction

Food industries have to satisfy the demand of the market for innovative food products and functional foods. For this purpose, more than 20 main hydrocolloids are used to modify textures and quality aspects during processing and cooking. Pectin is one of these food ingredients used for its gelling and stabilizing properties. They are mostly extracted from lemon peels and apple pomaces (May, 1990; Rolin, 2002). Pectins are polysaccharides composed mainly of α-D-1.4 linked galacturonic acid (GalA) chains (also called homogalacturonan or smooth regions) in which the carboxyl groups of the GalA can be free or methyl-esterified. Pectins are also constituted of hairy regions (also called rhamnogalacturonan I) with GalA-rhamnose regions where the rhamnose moieties can be substituted with neutral sugars (mainly arabinans and arabinogalactans) (Pilnik & Voragen, 1991; Voragen, Pilnik, Thibault, Axelos & Renard, 1995). Only small amounts of neutral sugars are present in commercial pectins as a result of the acid extraction (Guillotin et al., 2005; Kravtchenko, Voragen & Pilnik, 1992). Several types of pectins can be used as gelling agents in food since their gelling properties depend mainly on the nature of the substituents (methyl-esters, amide or acetyl groups) and the level of substitution. High methyl-esterified pectins (HM) with a degree of methyl-esterification (DM) of 50% or higher are distinguished from low methyl-esterified (LM) pectins with a DM up to 50% and low methyl-esterified and amidated (LMA) pectins. These pectins behave totally differently in food systems since HM pectins are mainly used in acid (pH below 3.5) and high sugar content products, while LM and LMA pectins are used in low sugar content at neutral and acidic pH and in the presence of calcium (Gilsenan, Richardson & Morris, 2000; May, 1990; Voragen et al., 1995). Low methyl-esterified amidated pectins (LMA) are obtained from HM pectins by amidation and partly de-esterification in a heterogeneous system in the presence of ammonia and alcohol (Anger & Dongowski, 1988) or in a homogeneous system using concentrated aqueous ammonia (Black & Smit, 1972). Not more than 25% of the total amount of carboxyl groups is allowed in the amide form for food products (Rolin & De Vries, 1990). Gels of LMA pectins have been compared to gels of methyl-esterified pectins with the same amount of free carboxy-groups and it was found that the higher firmness and strength of the LMA gel could be attributed to the presence of amide groups (Black & Smit, 1972). LMA pectins are used to achieve a better gelling control compared to low methyl-esterified pectins (LM) since they are less calcium sensitive than LM pectins and their gels are more thermoreversible (Racape,

Thibault, Reitsma & Pilnik, 1989). LM pectins are also used for the stabilisation of acid dairy drinks. The gelling mechanism of LMA pectins is not completely understood. Some authors stated that the "egg-box" model (Axelos, Thibault & Lefebvre, 1989; Grant, Morris, Rees, Smith & Thom, 1973; Thibault, Renard, Axelos, Roger & Crepeau, 1993; Thibault & Rinaudo, 1986) was not completely explaining the LMA pectins gels formation and it is known indeed that hydrogen bonds of amide groups are stabilizing the junctions zones as well (Alonso-Mougan, Meijide, Jover, Rodriguez-Nunez & Vazquez-Tato, 2002; Voragen et al., 1995).

In addition of the DM, the degree of amidation (DAm) is an important parameter to understand the different gelling behaviour of amidated pectins. To determine this DAm, food industries are using the titration method (Food Chemical Codex, 1981). The drawbacks of this method are the high amount of sample required, the non-specificity and the time needed to run the method. Another method used is infra-red spectroscopy and it was proven to be a useful and relatively quick tool to determine the DAm (Sinitsya, Copikova, Prutyanov, Skoblya & Machovie, 2000).

An alternative CE method has been developed to analyse pectic polymers according to their charge and to determine their DM (Jiang, Liu, WU, Chang & Chang, 2005; Jiang, Wu, Chang & Chang, 2001; Zhong, Williams, Goodall & Hansen, 1998; Zhong, Williams, Keenan, Goodall & Rolin, 1997). It is a fast method compared to FTIR and titration methods, accurate, and requiring very low amount of samples (nanoliters). These two last advantages are important in analytical studies, particularly for research purposes where sensitive methods using low amounts of samples are required since only low amounts of purified fractions are available.

Another benefit of CE is the possibility of the simultaneous analysis of polymers and oligomers in enzyme digests of pectins (Jiang et al., 2005; Jiang et al., 2001; Strom & Williams, 2004; Williams, Buffet & Foster, 2002; Williams, Foster & Schols, 2003; Zhong et al., 1998). This makes it possible to determine the distribution of methyl-esters over the galacturonan backbone by determining the degree of blockiness (DB) of commercial pectins with CE. The method commonly used to determine the DB has been described previously and is based on the analysis of the oligomers released after endo-polygalacturonase digestion of the pectins and their quantification using HPAEC at pH 5 (Daas, Meyer-Hansen, Schols, De Ruiter & Voragen, 1999; Daas, Voragen & Schols, 2000).

CE has not been explored yet as a tool to analyse the degree of amidation of pectins. A CE protocol was therefore developed in this study to determine the degree of substitution, the DAm and the DM of commercial pectins. The CE results are compared with the results obtained using the FTIR and titration methods. CE was also successfully used to determine the degree of blockiness of several commercial pectins and results were compared with the DB values determined using the HPAEC method as described previously.

2. Experimental

2.1. Samples

Pectin samples were kindly provided by Degussa texturant systems, Danisco and CP Kelco (Table I). Pectins C67, C56 and C30 have been obtained as described previously (Daas, Meyer-Hansen et al., 1999).

Pectins containing only amide groups were obtained by alkaline saponification. For this purpose, pectins (4 g) were dissolved in 500ml water at 40°C. An equal volume of 0.1 M sodium hydroxide was added in cold condition (4°C) to avoid β-elimination. After 24 hours, the samples were neutralised by adding 500 ml 0.1 M acetic acid. Pectins were then ultrafiltrated (Millipore Pellicon membrane; 10 kDa) and freeze dried. The saponified pectins are indicated with the denomination "sap" (e.g. Dsap, Gsap, O27sap-O5sap; Table I). The number associated to the code of some pectins corresponds to the DAm for amidated pectins and DM for methyl-esterified pectins.

2.2. Degree of methylation, degree of amidation of the pectins and uronic acid content of pectins used as references

The pectins obtained from CP Kelco, Danisco and Degussa texturant systems and used as references are characterised in Table I. The DAm and the DM of CP Kelco and Danisco pectins were determined by using the titration method (Food Chemical Codex, 1981). The first end-point of the titration corresponded to the amount of free carboxyl groups while the second end-point determined the amount of saponified carboxyl groups (since methyl-esters are saponified). The solution was then distillated and the distillate was titrated to determine the degree of amidation of the sample. The DAm of the samples provided by Degussa texturant systems was determined using the titration method, but the DM was estimated by

using the GC-headspace analysis of the free methanol released after alkaline de-esterification of pectins (Huisman, Oosterveld & Schols, 2004). The DM of the samples R70, C67, C56, CR52 and C30 was determined by HPLC as described previously (Daas, Meyer-Hansen et al., 1999).

The uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl method (Ahmed & Labavitch, 1977; Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979).

2.3. Determination of the DAm ,DM and DS by using FTIR method

Fourier Transform Infrared spectroscopy (FTIR) was performed on a Bio-Rad FTS 6000 spectrometer. At pH 6, methyl-esters, free carboxyl groups and amide groups have different wavelengths. Spectra were obtained with the detector MCT/DTGS set at 4 cm^{-1} resolution and 100 interferograms were collected to obtain a high signal to noise ratio.

The method for sample preparation was adapted from Chatjigakis et al., 1998: pectins (5 mg/ml) were dissolved in buffer phospate 0.02 M pH 6. Pectin solution (50 µl) was spread over the Fourier crystal and dried with a flush of air carefully onto the crystal surface. The integration (using PeakFit software; Aspire Software International) of each spectra area was obtained by using multiple gaussian decomposition of the characteristic bands in an IR spectrum in the region of 1800-1500 cm^{-1}. The peak area of the amide (absorption band at 1681 cm^{-1}) was divided by the sum of the peak area of the methyl-esters (absorption band at 1742 cm^{-1}) and the peak area of the free carboxyl groups (absorption band at 1611 cm^{-1}) to calculate the degree of amidation.

2.4. Determination of the DAM, DM and DS by using capillary electrophoresis

The analysis of the degree of amidation was adapted from the CE method developed previously to determine the DM (Zhong et al., 1998). Phosphate buffer 50 mM pH 7 was used as electrophoresis buffer. Samples and standards were wetted in 10 µl ethanol and dissolved in the buffer (5 mg/ml). At pH 7, pectins are fully ionised. Samples were analysed on an automated CE system (Beckman P/ACE MDQ) equipped with a UV Detector. A fused silica capillary internal diameter 50 µm, total length of 50.2 cm with 40 cm length capillary from inlet to detector, thermostated at 25°C was used. New capillaries were conditioned by rinsing for 15 min with 0.1 M NaOH, 30 min with distilled water and 30 min with phosphate buffer at 20 Psi. Between two runs the capillary was washed for 2 min with 0.1 M NaOH, 1min with

distilled water and 2 min with phosphate buffer at 20 Psi. All solutions were filtered over a 0.2 µm membrane. Detection was carried out by using UV absorbance at 190 nm with a bandwidth of 10 nm. Samples (50 µl) were loaded hydrodynamically (5 sec at 9.5 Psi) and electrophoresis was performed during 30 min in phosphate buffer pH 7 across a voltage of 20 kV for DS, DM and DAm analysis and 17 kV for the DB analysis and in normal polarity. L-methionine ethylester hydrochloride (0.044 mg/ml sample) was used as internal standard for CE in all samples.

The shift of the migration time of the internal standard, observed sometimes within a sample sequence, is corrected using the following transformation: $t_{cor} = 1/ [(1/t) –(x)]$, where t_{cor} is the migration time of the sample corrected from the internal standard shift, t is the migration time of the sample observed, x is the value to match the internal standard migration time for all samples.

The correlation of Electrophoretic Mobility (EM) with expected total charge was used for the determination of the degree of amidation. The equation to calculate the EM is described below:

$EM = EM_p - EM_m = (lL /V) [(1/t_p) – (1/t_m)]$

where EM_p corresponds to the observed mobility of the pectin and EM_m to the observed mobility of the internal marker, l is the distance from the inlet to the detector, L is the total length of the capillary, V the applied voltage, t_p and t_m are the migration times of the pectin and the internal marker respectively (Zhong et al., 1998).

2.5. Determination of the degree of blockiness of pectins with capillary electrophoresis

The determination of the degree of blockiness was adapted from the CE method developed previously for the separation of oligomers (Strom & Williams, 2004; Williams et al., 2002; Williams et al., 2003). Phosphate buffer 50 mM pH 7 was used as electrophoresis buffer. Pectin digests (5 mg/ml) were prepared as described previously (Guillotin et al., 2005) and dissolved in the buffer. Mono-, di- and triGalA (1 mg/ml buffer) were used as standards. L-methionine ethylester chloride (0.044 mg/ml sample) was added to pectin digests and standards as internal standard. Experiments were carried out on an automated CE system (Beckman P/ACE MDQ) equipped with a UV Detector as described previously for the determination of the degree of amidation. Samples (50 µl) were loaded hydrodynamically (5

sec at 0.5 Psi) and electrophoresis was performed using a voltage of 17 kV for 30 min in buffer (normal polarity).

3. Results and discussion

3.1. Determination of the degree of amidation of commercial pectins by using FT-IR.

FTIR spectroscopy was used to determine the DAm of pectins and spectra of LMA pectins and saponified LMA pectins were analysed in the range of 1500 to 1800 cm^{-1} corresponding to the most important region for our analysis (Figure 1).

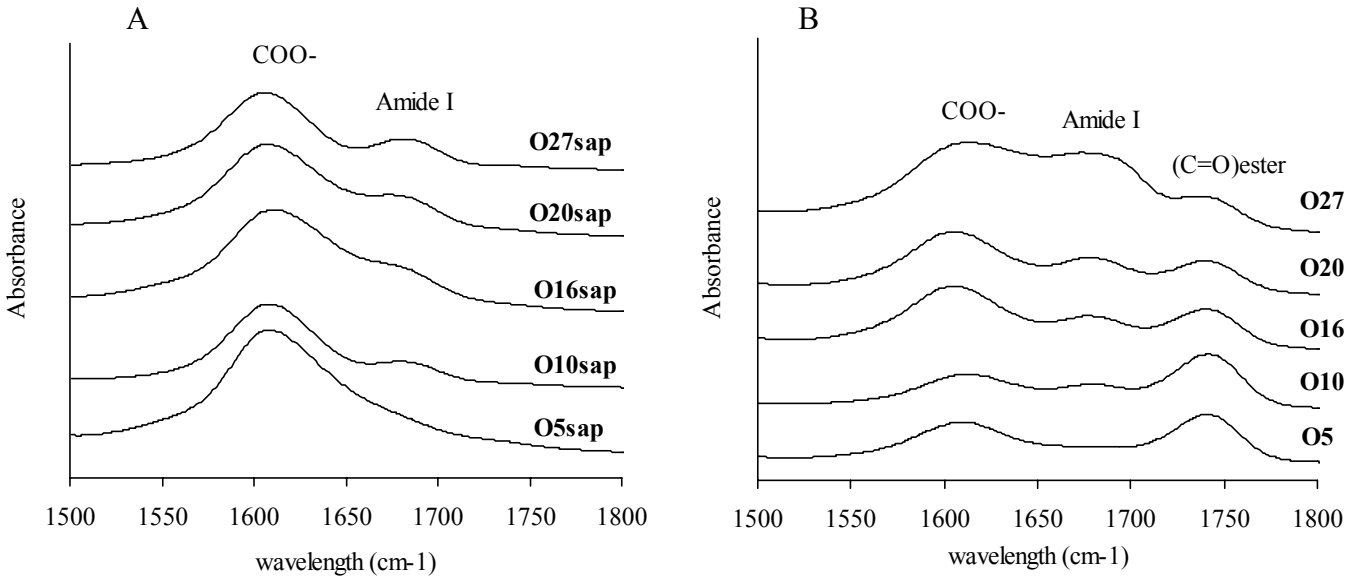

Figure 1: FITR spectra of saponified (A) and non-saponified (B) LMA pectins in the region of 1500-1800 cm^{-1}. Codes and characteristics of the pectins are explained in Table I.

The samples were analysed at pH 6. The pKa of pectins is in the range of 3.30-4.5 (Michel, Thibault & Doublier, 1984; Plaschina, Braudo & Tolstoguzov, 1978; Ravanat & Rindaudo, 1980). All free carboxyl-groups were in the ionized form at pH 6. In the 1500 to 1800 cm^{-1} region, the infra-red absorption by the carboxylic acid, the carboxylic methyl-esters groups and the primary amide groups (amide I) of pectin molecules are present (Bociek & Welti, 1975; Stewart & Morrison, 1992). Two main absorption bands were analyzed in our study for saponified amidated pectins (Figures 1A): one was observed around *1611* cm^{-1} and belonged to the a-symmetric stretching vibration of COO^-, the second absorption band was observed at

around *1681* cm^{-1} and corresponded to primary amide groups. When amidated pectins contained methyl-esters as well (Figures 1B), an additional band was present around *1742* cm^{-1} and belonged to the C=O vibration of methyl-esters groups. The positions of these three bands were similar to those found previously (Bociek & Welti, 1975; Chatjigakis et al., 1998; Sinitsya et al., 2000).

The degree of esterification is by definition the amount of methyl-esters (moles) present per 100 moles of total galacturonic acids (free GalA and substituted ones). It has been shown by Chatjigakis et al. (1998) that the ratio of the area of the band at 1742 cm^{-1} (methyl-esters) over the sum of the areas of the bands at 1742 cm^{-1} (methyl-esters), 1611 cm^{-1} (free GalA) and 1681 cm^{-1} (amide groups) was proportional to the DM as observed previously. The degree of amidation is the amount of amide groups divided by the total amount of GalA (free GalA + methyl-esterified and amidated ones) and it was as well found to be proportional to the area of the band at 1681 over the sum of the areas of the bands at 1742 cm^{-1}, 1611 cm^{-1} and 1681 cm^{-1} (Sinitsya et al., 2000).

By using the spectra decomposition described by Sinitsya et al, (2000), the comparison of the areas of peaks corresponding to free GalA, methyl-esterified GalA and amidated GalA gave DM and DAm values that did not correlate very nicely to the DAm and DM values obtained for our set of pectins as measured by titration. Therefore, peaks were integrated by using the commercial software peakfit as indicated in figure 2. A linear relationship between the DM determined by FTIR and the DM determined by titration (as used by the pectin manufacturers) was found with a high R-squared value ($R^2 = 0.97$; Figure 3).

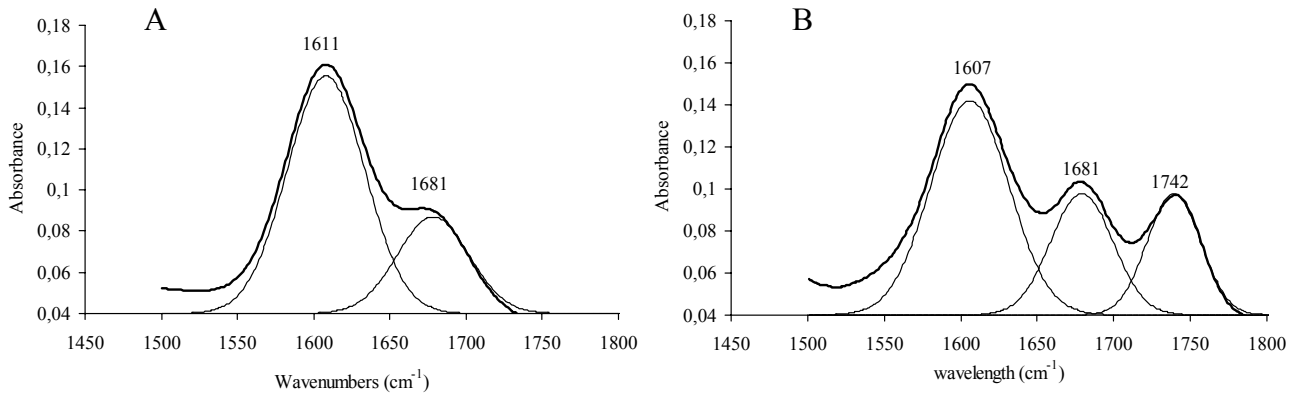

Figure 2: FITR spectra decomposition of saponified (A) and non saponified (B) LMA pectins O20 in the region of 1450- 1800 cm^{-1} using the peakfit software.

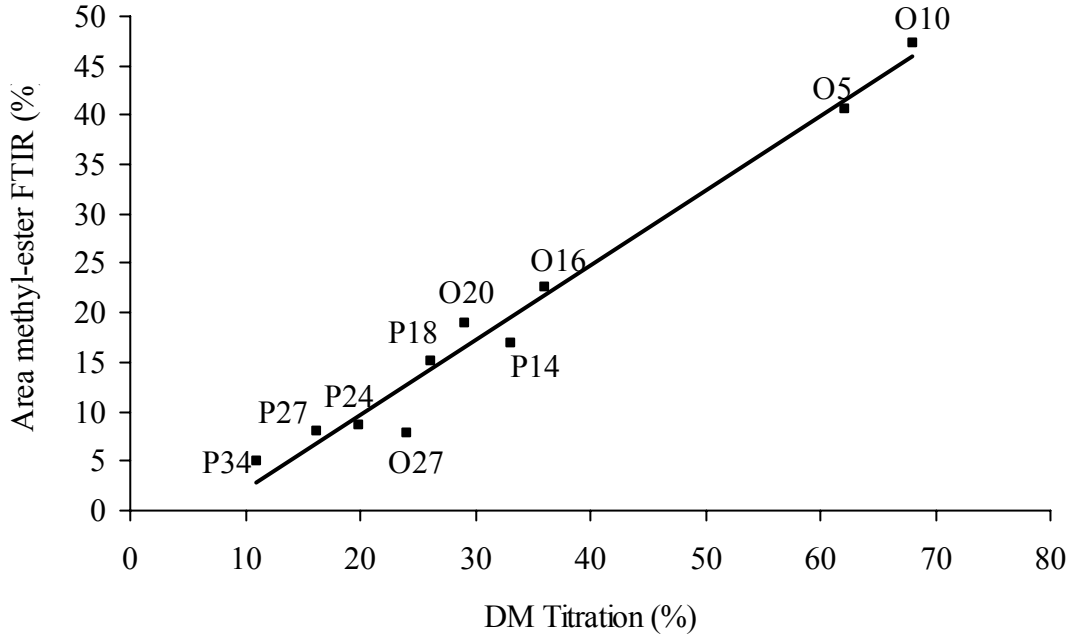

Figure 3: Correlation between DM values of low methyl-esterified amidated pectins obtained by using titration and FTIR [R^2 of 0.97].

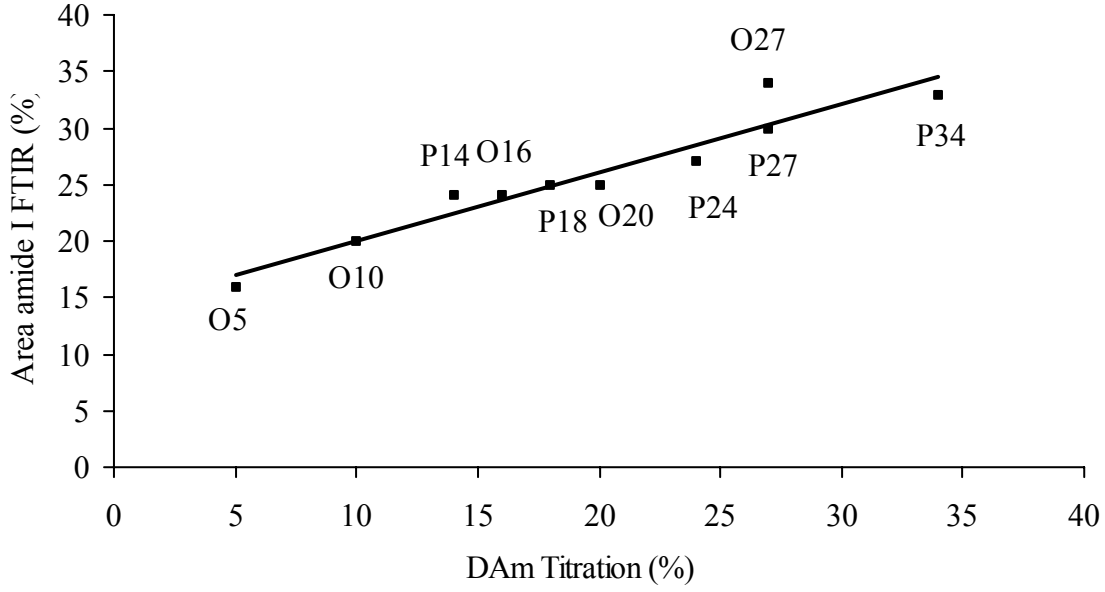

Figure 4: Correlation between DAm values of low methyl-esterified amidated pectins obtained by using titration and FTIR [R^2 of 0.92].

The same linear relationship was observed when the DAm was calculated from the FTIR spectrum and compared with the DAm values obtained using titration (R^2 = 0.92; Figure 4). The linear curve did not fit the origin as was observed before by Sinitsya et al. (2000).
The degree of substitution could be deduced easily from the DAm and DM since it corresponded to the sum of these two values. FTIR spectroscopy is an accurate method to determine DAm, DM and DS of pectins within one single FTIR spectrum. This method has also two main advantages for analytical research: it requires low amount of samples (0.25 mg of pectin) and it is easy to perform. However, the FTIR is rather time consuming since samples have to be analysed manually one by one after preparation of the pectin film on the FTIR crystal.

3.2. Determination of the degree of amidation of commercial amidated pectins by using capillary electrophoresis

3.2.1. Analysis of pectin standards

Since the determination of the DAm with the titration or FTIR methods needed rather large quantities (in the case of titration) and was rather time consuming as discussed previously, we searched for an alternative method. Capillary electrophoresis was introduced a few years ago to determine the DM of pectins (Zhong et al., 1998). The authors observed a linear relationship between the electrophoretic mobility (EM) of the pectin samples and the DM determined by titration. Zhong et al. showed no effect of the charge distribution of pectins on the electrophoretic mobilities (Williams et al., 2003; Zhong et al., 1997). Other authors claimed an effect of the intramolecular distribution of the methyl-esters (Jiang et al., 2001). However, in this last study (Jiang et al., 2001), only migration times were analysed and not electrophoretic mobilities, which makes it difficult to interpret the results and to compare them with those of Zhong et al., 2001 (Williams et al., 2003). In conclusion, the CE is considered to be not sensitive to the distribution of the substituents in contrast to the anion exchange chromatography as described previously (Guillotin et al., 2005).
For these reasons, CE might be a suitable method for the analysis of the degree of amidation of amidated pectins. For this reason, we adapted the CE method. Samples were analysed in phosphate buffer at pH 7 to obtain ionised free carboxyl groups and eletrophoregrams obtained are shown in Figure 5. The electrophoregrams were transformed as described in the

experimental section, to correct for the deviation of the internal standard peak (≈ 2.6 min). The electrophoretic mobility of the samples was determined. Less charged pectins are migrating faster to the cathode (where the detector is located) resulting in a higher mobility for HM pectins. Therefore HM pectins had a quicker mobility than the LM and LMA pectins (figure 5).

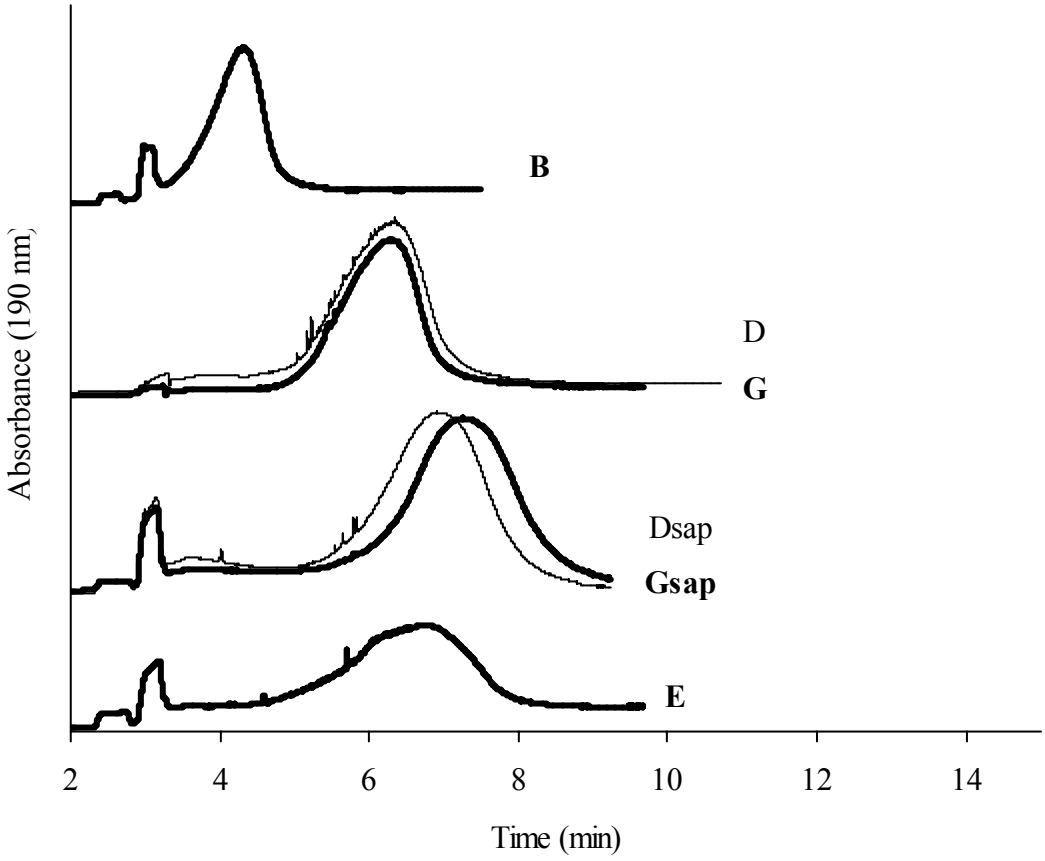

Figure 5: Electrophoregrams (transformed to correct for the deviation of the internal standard peak) of the pectin standards: HM pectin (B), LM pectin (E) and LMA pectins before (D and G) and after saponification of the methyl-esters (Dsap, Gsap).

The DS of the pectins was determined by using CE whereas the DM had been determined in this study by using the gas chromatography method. The DAm could be deduced after subtracting the DM values (obtained with GC headspace method) from the DS values (obtained with the CE method).

Another possibility to determine the DAm (and consequently the DS) of pectins is to saponify the samples to remove methyl-esters and to desalt them prior to their analysis using CE. The

DAm of pectins is not higher than 25%, which is the maximum allowed in food products. Therefore, commercial amidated pectins do not cover the whole range of DAm (up to 100 %). To get a wider range of substituted pectins for the calibration curve, methyl-esterified pectin were also used in addition to crude amidated pectins and saponified amidated pectins (figure 6). A linear relationship between the EM and the DAm/DS obtained by titration was found with a high *R*-squared value of 0.98 (Figure 6).

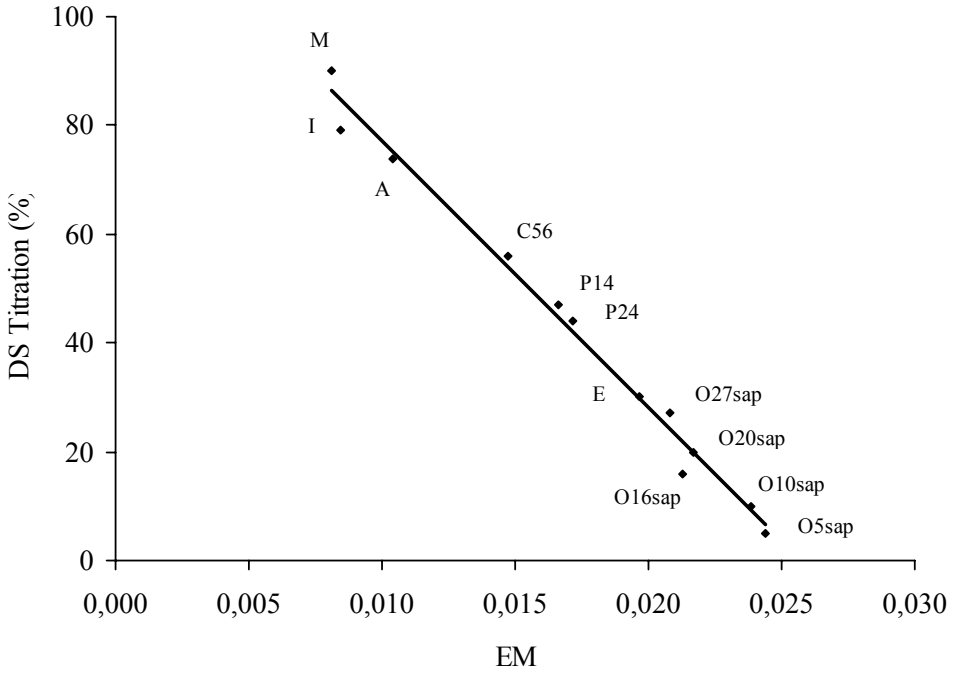

Figure 6: Linear regression of the electrophoretic mobility (EM) of commercial HM pectins, saponified and non saponified LMA pectins according to the DM and DAm (e.g. degree of substitution: DS) [$R^2 = 0.98$]. Codes for pectins included in the curve are explained in Table I.

Results were reproducible with small deviations (lower than 2%). Pectins having the same DM (L, H and J) but having a different distribution of the methyl-esters (DB of 6, 10 and 3%, respectively), showed a similar electrophoretic mobility (respectively, DM of 79, 80 and 79%). These results confirmed the previous findings where no effect of the charge distribution was observed by using CE (Williams et al., 2003; Zhong et al., 1997).

To check whether the presence of amide groups instead of a methyl-ester could modify the electrophoretic mobility of pectins, samples with similar DS but with and without amide groups were compared.

Table I: Characteristics of the pectin samples analysed.

pectins	supplier	GalA (%)	DM (%)	DAm (%) Titration	DS (%)	DS (%) CE
N	Degussa	84	81		81	79 ± 1.4
A	Degussa	82	74		*74*	*77* ± 1
B	Degussa	74	72		72	70 ± 0.7
M	Degussa	86	90		90	87 ± 0
L	Degussa	92	79		79	79 ± 0.7
H	Degussa	83	79		79	80 ± 0.7
J	Degussa	86	78		78	79 ± 0.4
F	Degussa	79	36		36	32 ± 0
E	Degussa	73	30		30	29 ± 0
D	Degussa	68	29	19	48	40
G	Degussa	70	31	18	49	41 ± 1
Dsap		71	0	19*	19*	26 ± 1
Gsap		69	0	18*	18*	22 ± 1.4
O27	Danisco	66	24	27	51	44 ± 1
O20	Danisco	71	29	20	49	48
O16	Danisco	77	36	16	52	49 ± 1.8
O10	Danisco	78	68	10	78	77 ± 0.7
O5	Danisco		62	5	67	68 ± 0
O27sap		63		27*	27*	23 ± 0
O20sap		68		20*	*20**	*19* ± 0
O16sap		69		16*	*16**	*20* ± 0.7
O10sap		74		10*	*10**	*9* ± 0.7
O5 sap		70		5*	*5**	*7* ± 1.4
P34	CP Kelco		11	34	45	44 ± 1.8
P27	CP Kelco		16	27	43	42 ± 1
P24	CP Kelco		20	24	44	41 ± 1
P18	CP Kelco		26	18	44	40 ± 1
P27b	CP Kelco		15	27	42	36 ± 0.7
P14	CP Kelco		33	14	47	44 ± 0.4
C67		81	67		67	75 ± 0
C56		79	56		56	54 ± 0
C30		79	30		30	30 ± 1

* DAm and DS were assumed to remain the same since all methyl-esters were removed by saponification as checked by using the gas chromatography.

The LMA pectin O16 (DS 52%) was compared with the HM pectin C56 (DS 56%; Table I) and it was found that the DS as measured by CE was quite similar (49% and 54%, respectively) while the levels of amidation and methyl-esterification were different.
These results suggested a similar effect of the amide groups and methyl-esters on the electrophoretic mobility of the pectins. The same phenomenon was observed when a LMA pectin and a HM pectin with similar DS were compared (pectins O10 and J; Table I). The CE method is thus a nice tool to analyse the DAm of amidated pectins.

3.2.2. Determination of the degree of blockiness of commercial pectins by using capillary electrophoresis and comparison with HPAEC pH 5 results

The CE method was used previously successfully to determine the DS, DAm and DM of pectins and was further used to analyse the distribution of substituents over the pectic backbone by determining the degree of blockiness of the non-methyl-esterified GalA. A high DB value indicates a blockwise distribution of the methyl-esters. Until now, the oligomers released after digestion of the pectins with an endo-polygalacturonase (endo-PG) were quantified by using HPAEC pH 5 in order to determine the DB (Daas, Meyer-Hansen et al., 1999; Daas et al., 2000). The charaterised oligomers present in the endo-PG digest pectin of a DM 30 pectin (Daas, Alebeek, Voragen & Schols, 1999; Limberg et al., 2000) were fractionated and analyzed again by CE to determine their position in the electrophoregram (Williams et al., 2002). The CE method was found to present two main advantages: the very low amount of sample required compared to the HPAEC method (Daas et al., 2000) and the simultaneous analysis of oligomers and polymers from the PG digest. Several digests of HM and LM pectins have been analyzed with CE to determine their degree of blockiness. The separation of oligomers was efficient (Figure 7).
Mono-, di- and trigalacturonic acid were quantified and the DB was calculated. The DB values obtained after separation of the oligomers by using CE have been compared with the DB values obtained after HPAEC elution of the oligomers (Table II) and were rather similar. The standard deviation of the DB obtained using the CE method was also comparable with the one obtained with the HPAEC method (0.2-2.7%). The CE method is thus an alternative method to determine the DB of pectins, which allows the use of very small amount of samples (10 times less than the amount needed for HPAEC).

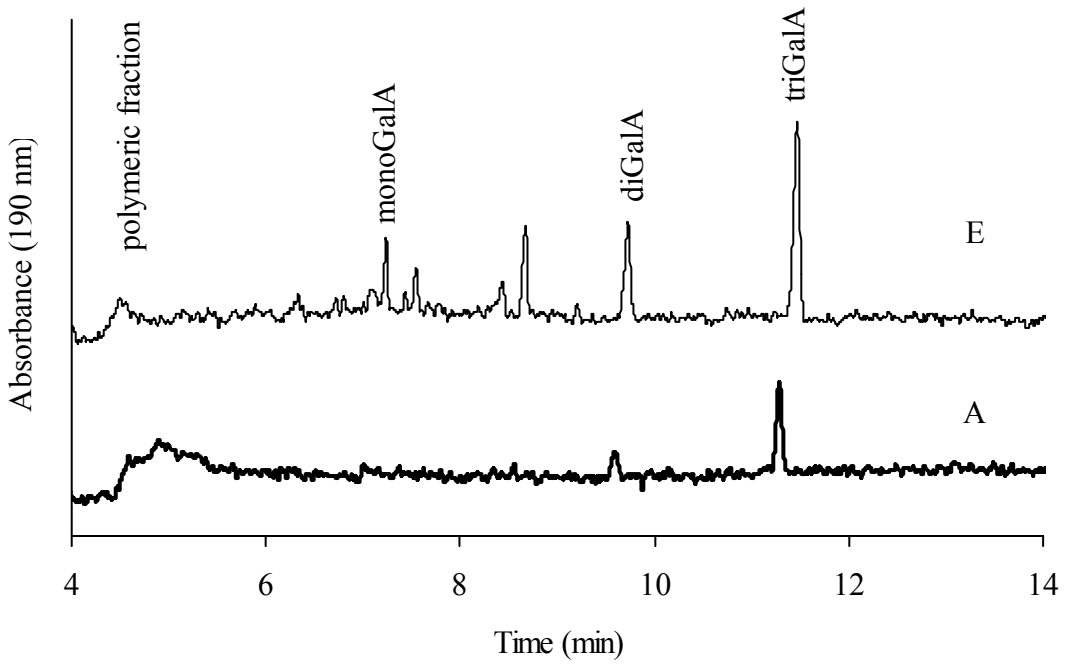

Figure 7: Electrophoregrams of HM pectin (A) and LM pectin (E) digested with endo-PG.

Table II: Comparison of the values for the degree of blockiness of pectins as found by CE and HPAEC.

Samples	GalA (w/w%)	DM (%)	DB CE (%)	DB HPAEC (%)
C	77.5	82	12	13
C56	79	56.1	9	8
C67	80.5	67.4	3	5
CR52	84.5	51.7	3	5
I	80.5	78.5	11	11
K	83.2	74.5	9	8
L	91.6	78.5	6	6
M	86.3	90	7	6
R70	79.2	70.2	1	1

Conclusions

The CE method is an accurate and fast method to determine the degree of amidation compared to the titration method and to the FTIR method since samples were analysed over night automatically without laborious sample pre-treatment procedures. It is now possible to characterise the DM, DS and DAm of pectins as well as the degree of blockiness by using only CE and thus very low amounts of samples (≈ 10 nl; 50 μg of pectin). This is very convenient in studies of the fine structure of pectins and pectin fractions. The CE method is a promising tool in the characterisation of pectins.

Acknowledgments: the authors would like to thank E. Ananta for her contribution in this work.

References

Ahmed A. E. R., Labavitch J. M. (1977). *Journal of Food Biochemistry*, *1*, 361-365.

Alonso-Mougan M., Meijide F., Jover A., Rodriguez-Nunez E., Vazquez-Tato J. (2002). Rheological behaviour of an amide pectin. *Journal of Food Engineering*, *55*, 123-129.

Anger H., Dongowski G. (1988). Amidated pectins - characterization and enzymatic degradation. *Food Hydrocolloids.*, *2*, (5), 371-379.

Axelos M. A. V., Thibault J.-F., Lefebvre L. (1989). Structure of citrus pectins and viscosimetric study of their solution properties. *Int. Journal Biol. Macromol.*, *11*, 186.

Black S. A., Smit C. J. B. (1972). The effect of demethylation procedures on the quality of low-ester pectins used in dessert gels. *Journal of Food Science*, *37*, (II), 730-732.

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

Bociek S., Welti D. (1975). The quantitative analysis of uronic acid polymers by infrared spectroscopy. *Carbohydrate Research*, *42*, 217-226.

Chatjigakis A. K., Pappas C., proxenia N., Kalantzi O., Rodis P., Polissiou M. (1998). FT-IR spectroscopic determination of the degree of esterification of cell wall pectins from stored peaches and correlation to textural changes. *Carbohydrate Polymers*, *37*, 395-408.

Daas P. J. H., Alebeek G. J. W. M. v., Voragen A. G. J., Schols H. A., (1999). Determination of the distribution of non-esterified glacturonic acid in pectin with endo-polygalacturonase. In: *Gums and Stabilisers for the food industry*; Williams P. A., Phillips G. O., Wrexham, The Royal Society of Chemistry, 3-18.

Daas P. J. H., Meyer-Hansen K., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research*, *318*, 135-145.

Daas P. J. H., Voragen A. G. J., Schols H. A. (2000). Characterisation of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research*, *326*, 120-129.

Food Chemical Codex, (1981) 3rd Ed., National Academy of Sciences, Washington, DC

Gilsenan P. M., Richardson R. K., Morris E. R. (2000). Thermally reversible acid-induced gelation of low-methoxy pectin. *Carbohydrate Polymers*, *41*, 339-349.

Grant G. T., Morris E. R., Rees D. A., Smith P. J. C., Thom D. (1973). Biological interactions between polysaccharides and divalent cations: the egg-box model. *FEBS Letters*, *32*, 195.

Guillotin S. E., Bakx E. J., Boulenguer P., Mazoyer J., Schols H. A., Voragen A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*, 391-398.

Huisman M. M. H., Oosterveld A., Schols H. A. (2004). New method for fast determination of the degree of methylation of pectins by headspace GC. *Food Hydrocolloids.*, *18*, (4), 665-668.

Jiang C. M., Liu S.-H., WU M.-H., Chang W.-M., Chang H.-M. (2005). Determination of the degree of esterification of alkaline de-esterified pectins by capillary zone electrophoresis. *Food Chemistry*, *91*, 551-555.

Jiang C. M., Wu M. C., Chang W.-H., Chang H.-M. (2001). Determination of random- and blockwise-type de-esterified pectins by capillary zone electrophoresis. *Journal of Agricultural and Food Chemistry*, *49*, 5584-5588.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992). Analytical comparison of three industrial pectin preparations. *Carbohydrate Polymers*, *18*, 17-25.

Limberg G., Korner R., Buchholt H. C., Christensen T. M. I. E., Roepstorff P., Mikkelsen D. J. (2000). Quantification of the amount of galacturonic acid residues in blocksequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger*. *Carbohydrate Research*, *327*, 321-332.

May C. D. (1990). Industrial pectins: sources, production and applications. *Carbohydrate Polymers*, *12*, 79-99.

Michel F., Thibault J.-F., Doublier J.-L. (1984). Viscosimetric and potentiometric study of high-methoxyl pectins in the presence of sucrose. *Carbohydrate Polymers*, *4*, (283),

Pilnik W., Voragen A. G. J., (1991). In Fox P. F. *Food Enzymology* (303-306). London: Elsevier Applied Science.

Plaschina I. G., Braudo E. E., Tolstoguzov V. B. (1978). Circular dichroism studies of pectin solutions. *Carbohydrate Research*, *60*, (1),

Racape E., Thibault J. F., Reitsma J. C. E., Pilnik W. (1989). Properties of amidated pectins II. Polyelectrolyte behavior and calcium binding of amidate pectins and amidated pectic acids. *Biopolymers*, *28*, 1435-1448.

Ravanat G., Rindaudo M. (1980). Investigation on oligo- and polygalacturonic acids by potentiometry and circular dichroism. *Biopolymers*, *19*, 2209-2222.

Rolin C., (2002). Commercial pectin preparations. In: *Pectins and their Manipulation*; Seymour G. B., Knox J. P., Blackwell Publishing Ltd., 222-239.

Rolin C., De Vries J. A., (1990). Pectin: Elsevier.

Sinitsya A., Copikova J., Prutyanov V., Skoblya S., Machovie V. (2000). Amidation of highly methoxylated citrus pectin with primary amines. *Carbohydrate Polymers*, *42*, 359-368.

Stewart D., Morrison I. M. (1992). FT-IR Spectroscopy as a tool for the study of biological and chemical treatments of barley straw. *Journal of the Science of Food and Agriculture*, *60*, 431-436.

Strom A., Williams M. A. K. (2004). On the separation, detection and quantification of pectin derived oligosaccharides by capillary electrophoresis. *Carbohydrate Research*, *339*, 1711-1716.

Thibault J.-F. (1979). Automatisation du dosage des substances pectiques par la méthode au meta-hydroxydiphenyl. *Lebensmittel Wissenschaft und Technologie*, *12*, 247-251.

Thibault J.-F., Renard C. M. G. C., Axelos M. A. V., Roger P., Crepeau M.-J. (1993). Studies of the lenght of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydrate Research*, *238*, 271-286.

Thibault J.-F., Rinaudo M. (1986). Chain association of pectic molecules during calcium-induced gelation. *Biopolymers*, *25*, 456-468.

Voragen A. G. J., Pilnik W., Thibault J.-F., Axelos M. A. V., Renard C. M. G. C., (1995). Pectins. In: *Food polysaccharides and their applications*; Stephen A. M., New York: Marcel Dekker Inc., 287-339.

Williams M. A. K., Buffet G. M. C., Foster T. J. (2002). Analysis of partially methyl-esterified galacturonic acid oligomers by capillary electrophoresis. *Analytical Biochemistry*, *301*, 117-122.

Williams M. A. K., Foster T. J., Schols H. A. (2003). Elucidation of pectin methylester distributions by capillary electrophoresis. *Journal of Agricultural and Food Chemistry*, *51*, 1777-1781.

Zhong H. J., Williams M. A. K., Goodall D. M., Hansen M. E. (1998). Capillary electrophoresis studies of pectins. *Carbohydrate Research*, *308*, 1-8.

Zhong H. J., Williams M. A. K., Keenan R. D., Goodall D. M., Rolin C. (1997). Separation and quantification of pectins using capillary electrophoresis. *Carbohydrate Polymers*, *32*, (1), 27-32.

Chapter 5

Degree of blockiness of amide groups as indicator for differences between amidated pectins.

To be submitted in Biopolymers as:
S.E Guillotin, J. Van Kampen, P.Boulenguer, , H.A. Schols and A. G. J. Voragen.

Abstract

Thickening and gelling properties of commercial amidated pectins depend on the degree of amidation and methyl-esterification, but also the distribution of these groups is of great importance. Methods have been developed during the last few years to determine the distribution of methyl-esters over the pectic backbone. We applied the strategies developed for the analysis of high methyl-esterified pectins for studying the distribution of amide groups in amidated pectins. Low methyl-esterified amidated (LMA) pectins were digested before and after removal of methyl-esters by an endo-polygalacturonase to determine the degree of blockiness of the substituents. The nature of the substituents (amide groups compared to methyl-esters) did not modify the behavior of the enzyme. Oligomers released were separated by using high-performance anion exchange chromatography at pH 5. Fractions collected after on-line desalting were identified by using MALDI-TOF mass spectrometry. Oligomers were found to elute from the column as function of their total charge. For the same overall charge and size, oligomers with methyl-esters eluted before oligomers with amide groups. Both amide groups and methyl-esters of the LMA pectins studied were found to be semi-randomly distributed over the pectic backbone, but this may vary according to the amidation process used.

1. Introduction

Pectins are used in the food industry for their gelling, thickening and stabilizing properties. Pectins are mainly composed of α-D-1.4 linked galacturonic acid residues (GalA) (Barrett & Northcote, 1965; De Vries, Voragen, Rombouts & Pilnik, 1981). In nature, the carboxyl groups on C-6 of the GalA residus can be free or methyl-esterified. Native pectins also contain regions of GalA-rhamnose sequences to which most of the neutral sides chains are attached (Darvill, McNeill & Albersheim, 1978; McNeill, Darvill & Albersheim, 1980; Neukom, Amado & Pfister, 1980; Pilnik & Voragen, 1991; Voragen, Pilnik, Thibault, Axelos & Renard, 1995). As a result of the acid extraction of pectins (mainly from lemon peels), the neutral sugar (NS) content of pectins is really low (5-10% in w/w%) (Guillotin et al., 2005; Kravtchenko, Voragen & Pilnik, 1992a; Lecacheux & Brigand, 1988). The different gelling properties of the extracted material depend on many factors (e.g. varieties of lemons, growing, harvesting and processing conditions). At a molecular level, the physical properties of pectins are influenced by the molecular weight (Christensen, 1954; Owens, Svenson & Schultz, 1933; Van Deventer-Schriemer & Pilnik, 1987), the degree of substitution, the nature and the distribution of the substituents (randomly or blockwise distributed over the GalA backbone) (Lofgren, Guillotin, Evenbratt, Schols & Hermansson, 2005; Powell, Morris, Gidley & Rees, 1982; Rolin, 2002; Thibault & Rinaudo, 1986; Voragen et al., 1995). Commercial pectins are classified in high methyl-esterified pectins also called HM pectins (degree of methyl-esterification higher or equal to 50%) and low methyl-esterified pectins also called LM pectins (degree of methyl-esterification below 50%). HM pectins are amidated for a better control of their gelling behavior. Furthermore low methyl-esterified amidated pectins (LMA) give more thermoreversible gels, need less calcium to gel when compared to LM pectins with similar degree of substitution (Black & Smit, 1972; Racape, Thibault, Reitsma & Pilnik, 1989) and their gels are stronger below pH 3 compared to LM pectins (Lootens et al., 2003).
Commercial amidated pectins with similar chemical characteristics (molecular weight, galacturonic acid and neutral sugar content, degree of methyl-esterification and degree of amidation) have different gelling properties in the presence of calcium. These differences in physical behavior may be due to a different distribution of methyl-esters and/or amide groups. A way to characterise differences in distribution of these substituents is to establish the degree of blockiness (DB) of both methyl-esters and amide groups. The method to determine the degree of blockiness of methyl-esters has been described previously (Daas, Meyer-Hansen,

Schols, De Ruiter & Voragen, 1999; Daas, Voragen & Schols, 2000). Methyl-esterified pectins were digested with an endo-polygalacturonase (endo-PG) obtained from *Kluyveromyces fragilis* and degradation products were analyzed and quantified using HPAEC pH 5. All fully and partially methyl-esterified oligogalacturonides observed in the elution pattern were previously identified by using Maldi-TOF mass spectrometry (Daas, Arisz, Schols, De Ruiter & Voragen, 1998; Daas et al., 1999; Daas et al., 2000). The amount of mono, di- and trigalacturonic acid released by the enzyme compared to the amount of free GalA presents in the sample was used to calculate the DB. A high DB value is indicative for a blockwise distribution of non-esterified galacturonic acid residues in pectins. The ratio of non-methyl-esterified oligomers versus methyl-esterified oligomers is also important for the characterisation of pectins since it indicates whether the PG degradable blocks are closer to each other or distant. In this study, all the degradation products present in the PG digest of amidated pectins were identified by using off-line coupled HPAEC-MALDI-TOF mass spectrometry. The method was applied to study differences in the distribution of methyl-esters and/or amide groups in commercial LMA pectins having similar chemical characteristics but different calcium sensitivity.

2. Material and methods

2.1. Pectins samples

Pectins were kindly provided by Degussa Texturant Systems, Danisco and Copenhagen Pectins (Table I). The galacturonic acid content (GalA) was determined by using the automated colorimetric *m*-hydroxydiphenyl method (Guillotin et al., 2005), the degree of methyl-esterification (DM) by using GC headspace method (Guillotin et al., 2005; Huisman, Oosterveld & Schols, 2004). The degree of amidation of these pectins was determined by the pectin manufacturer by using the titration method (Food Chemical Codex, 1981). All information about these pectins was summarized in Table I.

2.2. Saponification of the pectins

Pectins containing only amide groups were obtained by alkaline saponification. For this purpose, pectins (4 g) were dissolved in 500 ml water at 40°C. An equal volume of 0.1 M sodium hydroxide was added in cold condition (4°C) to avoid β-elimination. After 24 hours,

the samples were neutralised by adding 500 ml 0.1 M acetic acid. Pectins were then ultrafiltrated (Millipore Pellicon membrane; 10 kDa) and freeze dried. The saponified pectins are indicated with the denomination "sap" (Table I).

2.3. Analysis of oligomers and determination of the degree of blockiness by HPAEC pH 5 equipped with a PA1 column.

Pectins were digested with an endo-polygalacturonase (endo-PG) obtained from *Kluyveromyces fragilis* as described previously (Guillotin et al., 2005). Oligomers released upon PG treatment of the pectins were analysed by HPAEC (100 µl of 5 mg/ml digests) equipped with a Dionex CarboPac PA1 anion exchange column (250 × 2 mm) and a CarboPac PA-1 pre-column (50 × 2 mm). The column was equilibrated with 0.01 M sodium acetate pH 5 during 10 min. Elution was performed in two steps: from 0.01 to 0.55 M of sodium acetate pH 5 in 40 min and from 0.55 M to 1 M sodium acetate pH 5 in 60 min with a flow of 0.2 ml/min. The gradient was hold at 1 M sodium acetate pH 5 for 10 min. The PAD detector (Dionex) was equipped with a gold working electrode and an Ag/AgCl reference electrode. Detection of the oligomers was possible after post column addition of sodium hydroxide (1 M NaOH; 0.2 ml/min).

After the detector, two desalting units (Dionex) were connected in series: the ultra-self-regenerating anion suppressor 4 mm-unit (ASRS) was connected first to exchange the sodium ions for hydronium ions (H_3O^+). In addition, an ultra-self-regenerating cation suppressor 4 mm-unit (CSRS) was installed in series to exchange the acetate ions for hydroxide ions (OH^-). The continuous desalting of the eluent was achieved by the electrolysis of deionized water (8 ml/min) in both suppressors. Fractions (120 µl) were collected in a 96-well-plate equipped with filter (Millipore; 1.2 µm hydrophilic), using a Gilson FC-203B fraction collector.

The DB is the amount of mono- di- and trigalacturonic acid released by the endo-polygalacturonase related to the amount of free GalA present in the sample. The absolute degree of blockiness (DB_{abs}) is the amount of mono- di- and trigalacturonic acid released by the endo-polygalacturonase related to the total amount of GalA (free and methyl-esterified GalA) present in the sample (Guillotin et al., 2005).

2.4. Characterisation of oligomers by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) after HPAEC pH 5 elution

Fractions (120 µl) were desalted by using H^+-Dowex AG 50 WX8. Fractions were then filtered (1.2 µm hydrophilic filter to remove the H^+- Dowex) and collected in a second 96-well plate by using a vacuum pump. The desalted fractions were applied (1 µl) on the Maldi sample plate. On the top of this layer, 1 µl of matrix solution was added (dihydroxybenzoic acid (9 mg/ml) dissolved in 50% (v/v) acetonitrile).

MALDI-TOF MS analysis in the reflector mode was performed by using an Ultraflex instrument (Bruker Daltonic's) equipped with a nitrogen laser of 337 nm. The mass spectrometer was selected for positive ions. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12 kV. Hereafter, the ions were detected in the reflector mode. The lowest laser power required to obtain good spectra was used and at least 100 spectra were collected. The MALDI-TOF MS was externally calibrated with a mixture of oligoGalA.

3. Results and discussion:

3.1. Separation of oligomers obtained after enzymatic degradation of amidated pectins by endo-PG

The relation between chemical structure and functionality of two LMA pectins (D and G) has not been understood so far. The pectins had different gelling properties in the presence of calcium, while the chemical characteristics (GalA content, the DM, the DAm) were quite similar (Table I). The NS content of both LMA pectins is low (5% w/w). A different distribution of the substituents (methyl-esters and/or amide groups) may explain the different physical behavior of these two pectins. Therefore, we aimed to determine the degree of blockiness of these substituents as published for the determination of the DB of HM and LM pectins (Daas et al., 1999; Daas et al., 2000).

Table I: Galacturonic acid content, degree of methyl-esterification, degree of amidation of the samples, degree of blockiness and degree of blockiness absolute of amidated pectins and LM pectins.

Samples	Provider	GalA (w/w%)	DM	DAm	DS (%)	DB (%)	DB_{abs} (%)
O5	Danisco	70	62	5	67	12 ±2.2	3.9±0.7
O10	Danisco	78	68	10	78	10.4 ±0.1	2.3±0
O16	Danisco	77	36	16	52	9.2 ±0.8	4.4±0.4
O20	Danisco	71	29	20	49	9.6 ±0.1	4.9±0
O27	Danisco	66	24	27	51	10 ±1.2	4.9±0.6
O5sap	Danisco	70		5	5	14.8	14.1
O10sap	Danisco	74		10	10	13.6 ±1.3	12.2±1.1
O16sap	Danisco	69		16	16	12.4 ±0.6	10.4±0.5
O20sap	Danisco	68		20	20	11.9 ±2	9.5±1.6
O27sap	Danisco	63		27	27	11.4 ±0.4	8.3±0.3
P5	CPkelco	72	33	14	47	10.4	5.5
P18	CPkelco	66	26	18	44	10 ±1.8	5.6±1
P24	CPkelco	69	20	24	44	7.8 ±3	4.4±1.7
P27	CPkelco	72	15	27	42	8.6 ±0	5.0±0
P34	CPkelco	62	11	34	45	8.2 ±1.7	4.5±0.9
D	Degussa	68	29	19	48	7.6 ±3.4	3.9±1.8
G	Degussa	70	31	18	51	9 ±2.9	4.4±1.4
Dsap	Degussa	71	0	19	19	7.6 ±1.3	6.1±1
Gsap	Degussa	69	0	18	18	9.1	7.5
C30	Copenhagen Pectin	79	30		30	14.5 ±3.1	10.1±2.2

This DB method is based on the digestion of the pectins with an endo-PG and the analysis of the oligogalacturonides by using anion exchange chromatography equipped with a PA1 column (4 × 250 mm). This column was used in this study as well to analyse endo-PG digests obtained from amidated pectins. However, an efficient separation was not obtained between 10 and 20 min (results not shown). Another PA1 column with smaller dimensions (2 × 250 mm) showed a higher resolution towards the oligogalacturonides (Figure 1).

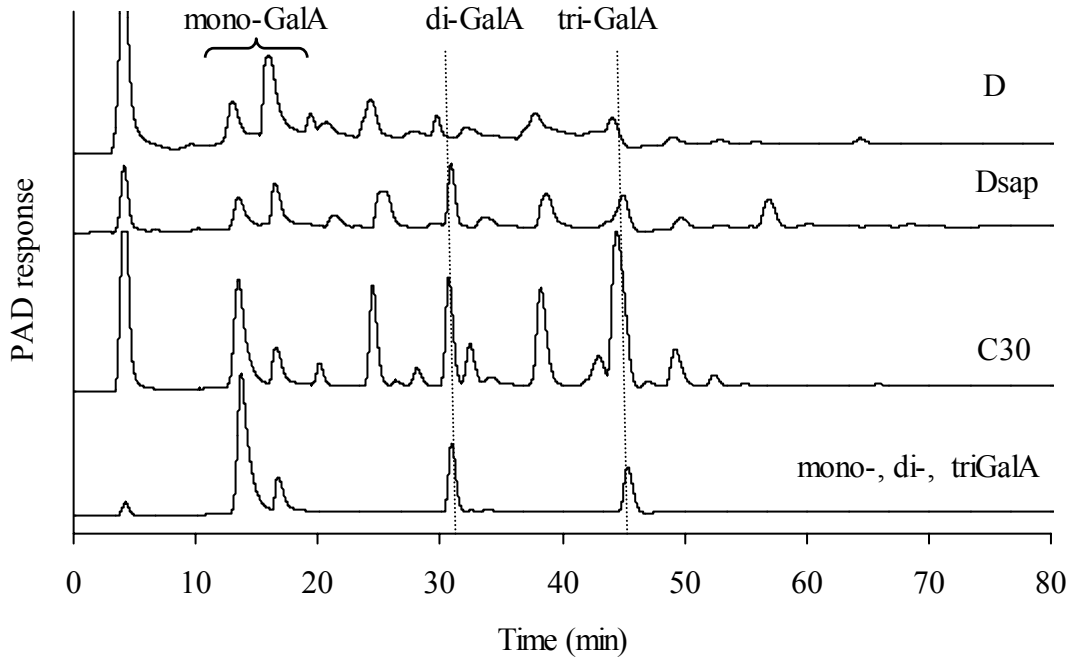

Figure 1: HPAEC pH 5 elution profiles (with post column sodium hydroxide addition) of a standard sample (mono-, di- and triGalA) and PG digests of the LM pectin (C30), the non-saponified amidated pectin (D) and the saponified amidated pectin (Dsap).

More oligomers were released when LMA pectin D was digested compared to the LM pectin C30 as a result of the double substitution of LMA pectins (Figure 1). Due to the high number of fragments, the separation of the oligomers present in pectin D digest was not as efficient as the separation of the pectin C30 digest. To identify all oligomers and to focus first on amide groups only, the LMA pectin D was saponified (Dsap). Consequently, less oligomers were observed in the pectin Dsap digest compared to the pectin D digest (Figure 1). Peaks were broader compared to those observed for pectin C30 which may be explained by the presence of oligomers having the same size and total charge but different distribution of the substituents over the oligomers.

3.2. Characterization of amidated oligomers from saponified and non-saponified LMA pectins

3.2.1. Analysis of the PG digest from saponified amidated pectin (Dsap)

The MALDI-TOF mass spectrum of the digest of pectin Dsap was shown in figure 2. As a result of the presence of many matrix peaks in the mass region up to 350 Da, mono- and di-GalA or their sodium or potassium adducts could not be distinguished.

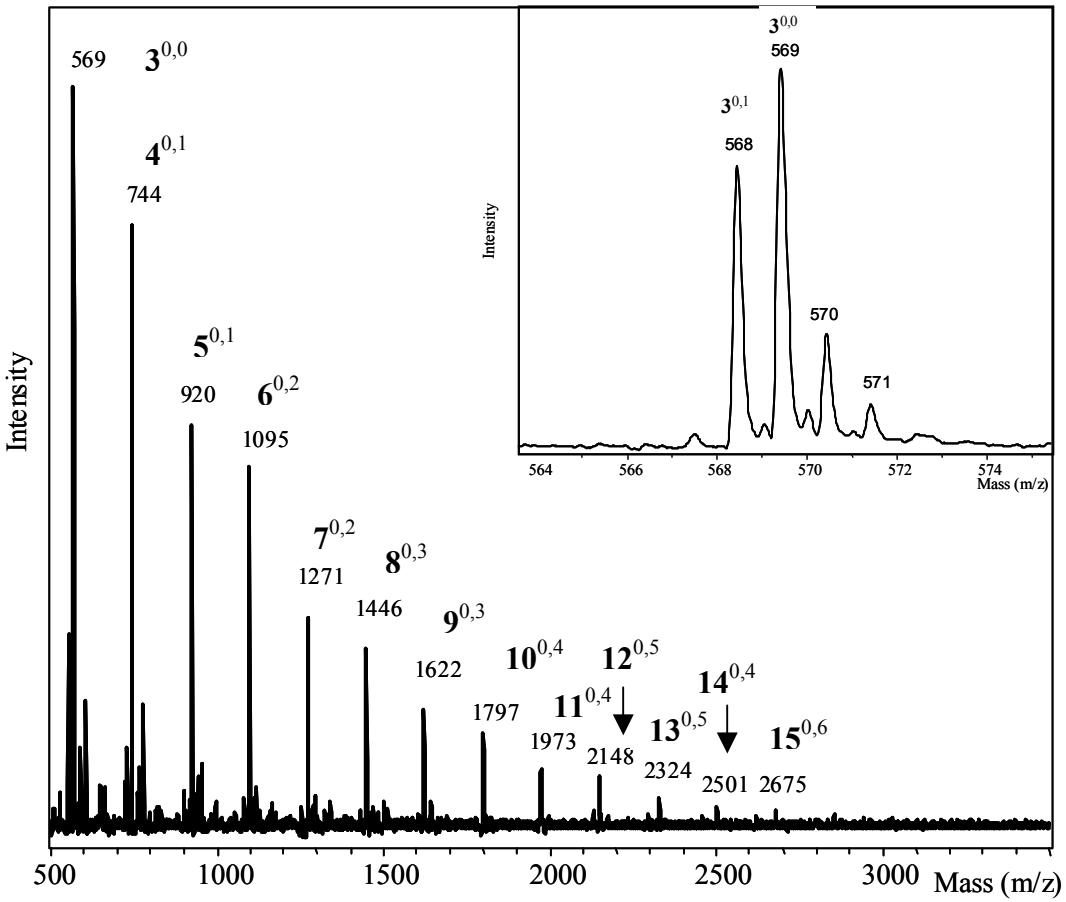

Figure 2: Maldi-TOF mass spectrum (positive mode) of the endo-PG digests of a saponified pectin D (Dsap). The molecular weight (Da), the DP (bold number), the number of methyl-esters (first superscript number) and amide groups (second superscript number) of a selected number of peaks are shown. A zoom of the triGalA mass range is inserted.

The size of the oligomers from pectin Dsap was indicated in Arabic numbers while the number of amide groups per oligomer was indicated in superscript (second number). The first number in superscript indicated the amount of methyl-esters and these substituents were

absent in Dsap as a result of the saponification. TriGalA without substituents ($\mathbf{3}^{0,0}$) was detected as a sodium adduct (m/z 569) and the triGalA oligomer with one amide group ($\mathbf{3}^{0,1}$) was detected as well (m/z 568; insert of Figure 2).

There is only one Da of difference between the oligomer without amide group ($\mathbf{3}^{0,0}$) and with one amide group ($\mathbf{3}^{0,1}$). The two extra peaks at 570 and 571 Da (insert of Figure 2) corresponded to the isotopes of the triGalA ($\mathbf{3}^{0,0}$; 569 Da). The peak 569 was higher than peak 568 due to C^{13} isotopes of the oligomer of 568Da (Alebeek, Schols & Voragen, 2001).

3.2.2. Characterisation of the PG oligomers from pectin Dsap after separation on HPAEC at pH5

Oligomers from the Dsap digest separated on the PA1 column have been collected after desalting. The run was performed in the absence of post column sodium hydroxide addition and PAD detection to avoid saponification of the oligomers. No elution profile could therefore be recorded but the separation of the oligomers was checked by injecting the same sample with post column addition before and after the fractionated run. The two elution profiles recorded were precisely the same (Figure 3).

Fraction of 120 µl were pooled and analysed by using MALDI-TOF MS. The MALDI-TOF mass spectrum of the fraction F44 (26.4 min) was shown in figure 4 as an example. In this fraction, only one oligomer was observed (apart from the matrix peaks) corresponding to the sodium adduct of a triGalA with one amide group ($\mathbf{3}^{0,1}$; 568 Da). The two others main peaks (590 and 612 Da) corresponded to the sodium salts of the sodium adduct of this oligomer (568 +22 and 568 +44). The complete sequence of elution of the oligomers from the endo-PG digest of Dsap at pH5 (figure 3) was determined from the MALDI-TOF MS results.

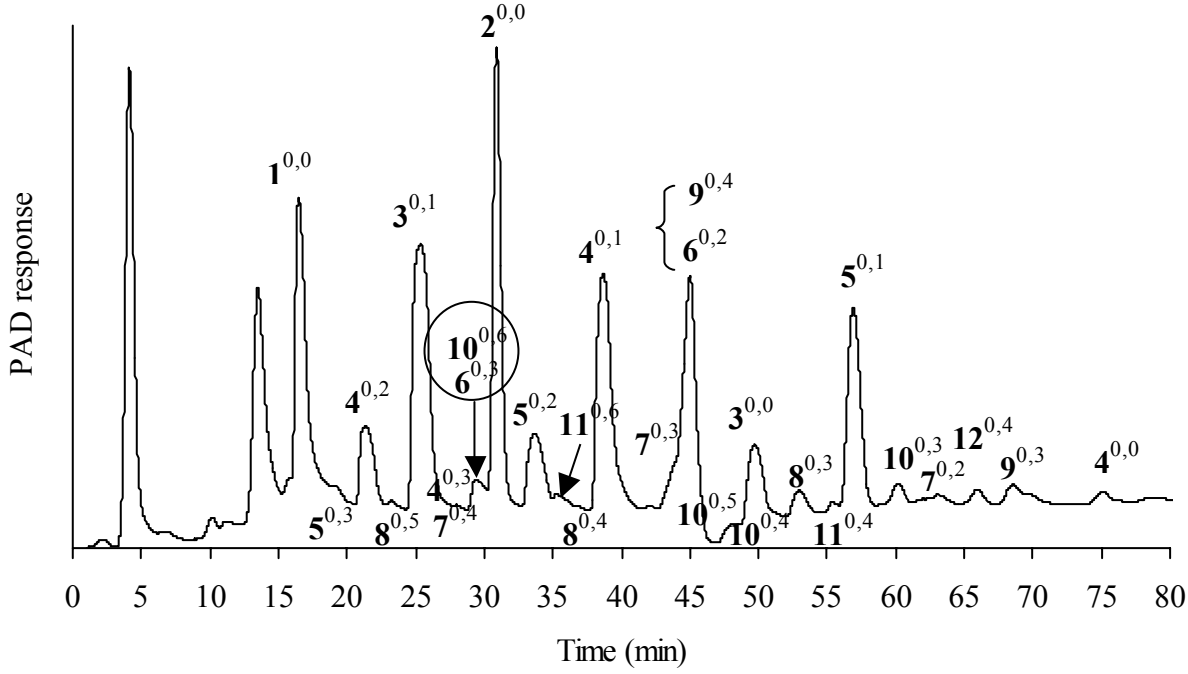

Figure 3: HPAEC pH 5 elution profiles (with post column sodium hydroxide addition) of pectin Dsap digested by endo-PG. Fractions (120 µl) were collected and analysed with Maldi-TOF MS. The DP (bold number), the number of methyl-esters (first superscript) and the number of amide groups (second superscript) of the oligomers identified with MALDI-TOF MS are shown.

In figure 5, the elution time of the oligomers was compared to their total charge at pH 5. This schematical representation indicated that the elution of amidated oligomers was depending on their overall negative charge. The free monoGalA (one charge) was eluted before the GalA oligomer of DP 5 with two negative charges: so the more charges, the later the elution. However, the total charge was not the only criteria of the elution on the PA1 column at pH 5. The binding of oligomers was following two other rules. Firstly, for oligomers with the *same degree of polymerization,* the charge influenced the elution behavior: for DP 5 amidated oligomers, the one with less free carboyl groups ($\mathbf{5}^{0,3}$) eluted before the one with more free carboxyl groups ($\mathbf{5}^{0,2}$). Secondly, for oligomers having the *same charge*, the size dictated the elution behaviour: for a total charge of 3, the oligomer with a DP 8 eluted before the one with a DP of 7. These results clarify the complex elution behavior of endo-PG digests from saponified amidated pectins over the PA1 column at pH 5.

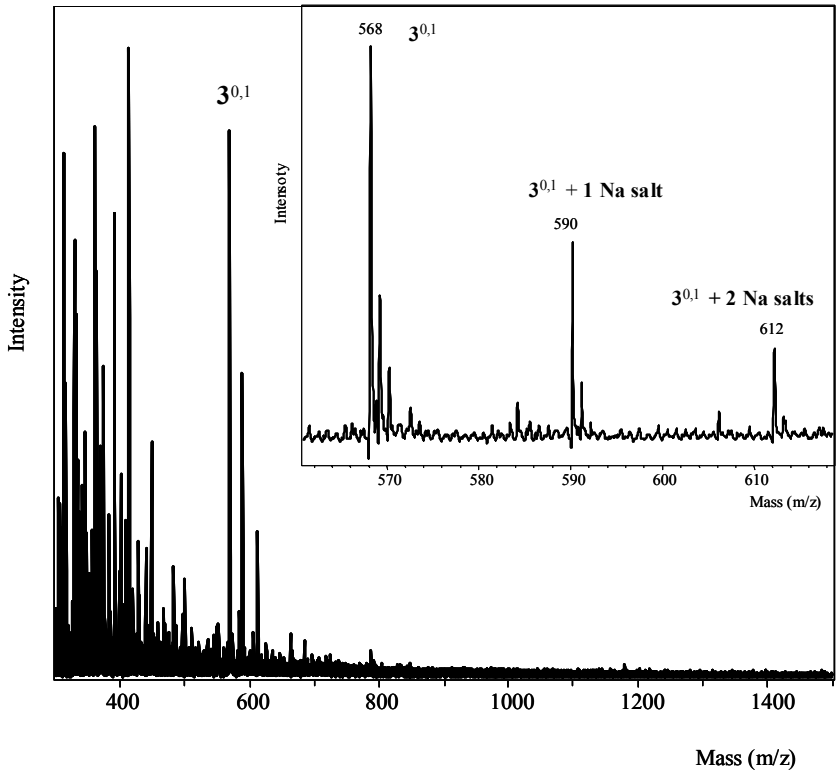

Figure 4: Maldi-TOF mass spectrum (positive mode) of the peak eluting at 26 min (after injection of Dsap endo-PG digest on the anion exchange column). The molecular mass (m/z) and the DP (bold number) are shown. The first number in superscript denotes the number of methyl-esters and the second number in superscript denotes the number of amide groups. Peaks with masses below 500 m/z are matrix peaks. A zoom of the triGalA is inserted.

It was interesting to note that amidated oligomers followed the same principle of elution behavior as described by Daas et al. for methyl-esterified oligomers (Daas et al., 1998), although, minor differences in the elution behavior existed when an amide group was present instead of a methyl-ester.

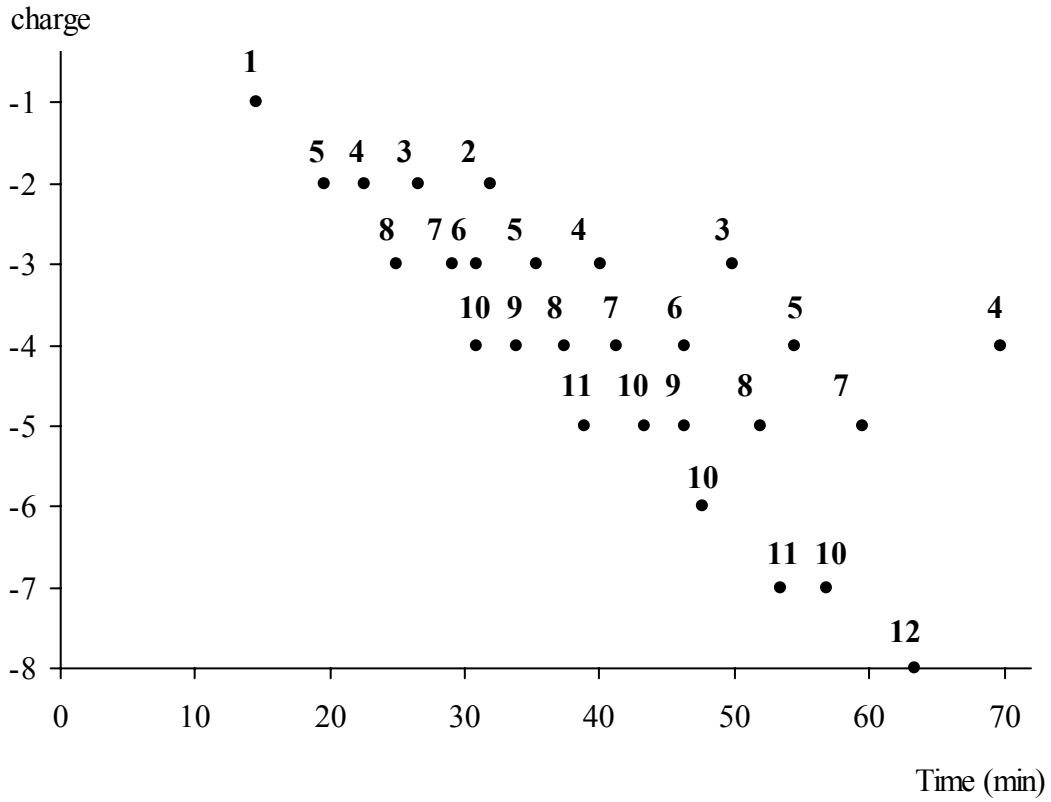

Figure 5: Schematical representation of the elution time of oligomers as function of their charge and DP after separation on HPAEC at pH 5. Oligomers were obtained after endo-PG digestion of the saponified pectin D (Dsap). Arabic numbers indicate the DP.

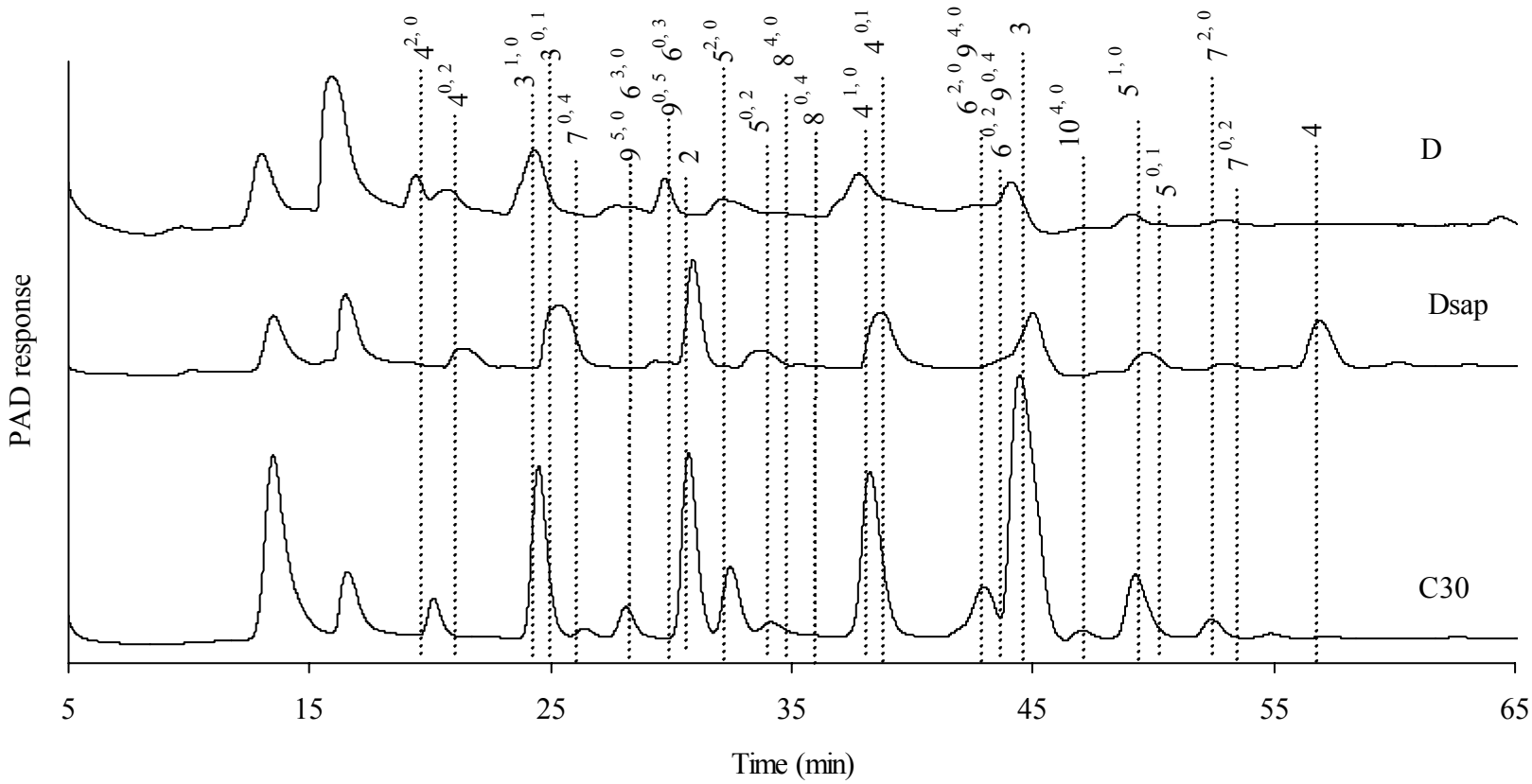

Figure 6: HPAEC pH 5 elution behaviour of partly amidated AND methyl-esterified galacturonic acid oligomers present in an endo-PG digest of LMA pectin D. These oligomers are also compared to those obtained from the PG digests of LM pectin C30 and the saponified LMA pectins Dsap. The arabic number indicates the DP. The first number in superscript denotes the number of methyl-esters and the second number in superscript denotes the number of amide groups.

3.2.3. Characterisation of the endo-PG oligomers from pectin D after separation on HPAEC at pH 5

The digest from pectin D (with amide groups and methyl-esters) was also identified with MALDI-TOF MS (Figure 6). It was interesting to note that for the same DP and overall charge, an oligomer with a methyl-ester eluted before an oligomer with an amide group: a trimer of GalA with an methyl-ester group ($\mathbf{3}^{1,0}$) eluted before a trimer of GalA with an amide group ($\mathbf{3}^{0,1}$) as indicated in Figure 6. We speculated that this might be explained by the higher steric hindrance of a methyl-ester, which would decrease the interaction of a neighboring carboxylate group with the anion exchanger compared to an amide group, *or*, to the weaker binding on the anion exchanger of $\mathbf{3}^{1,0}$ compared to $\mathbf{3}^{0,1}$ as a result of the slightly higher polarity of the amide group compared to a methyl-ester.

3.3. Degree of blockiness of amide groups from saponified and non saponified LMA pectins

Since all peaks observed in the elution patterns of the amidated pectins digests at pH 5 were characterized, the degree of blockiness of several LMA pectins and saponified LMA pectins was calculated (Table I). A high DB value is indicative for a blockwise distribution of non-substituted galacturonic acid residues in pectin (Daas et al., 1999; Daas et al., 2000).

3.3.1. Polygalacturonase behaviour towards amide groups on pectins

The endo-polygalacturonase used needs at least 4 free GalA to degrade the methyl-esterified pectins (Pasculli, Geraeds, Voragen & Pilnik, 1991). It was assumed in our study that endo-PG acted in the same way towards methyl-esters and amide groups. This assumption was based on the fact that saponified amidated pectins (DAm of 20 %) were degraded by endo-PG in a rather similar way as a LM pectin with a similar DS (DM17%; C17) (Daas, Boxma, Hopman, Voragen & Schols, 2001) as monitored by HPSEC (results not shown). The pectins Dsap and Gsap were slightly less digested by the endo-PG compared to the pectin C17 but this may be due to a different distribution of the substituents, since pectin C17 was found to have a blockwise distribution of the methyl-esters (DB of 38.9%). A similar sensitivity of the endo-PG for methyl-esters and amide groups was also found by comparison of the DS 50 pectins (LM and amidated pectins). This enzyme activity was confirmed by other studies

where the degradation of both LMA and LM pectins with endo-PG and pectin esterase was compared (Anger & Dongowski, 1988).

3.3.2. Distribution of amide groups over the galacturonic acid backbone

The degree of blockiness of several amidated pectins differing in the amount of methyl-esters and amide groups were studied to analyse the distribution of the amide groups and/or methyl-esters. The standard deviation of the DB and DB_{abs} values of all the samples analysed was low (respectively, 0-3.4% and 0-2.2%). The DB_{abs} values give information about the absolute number of PG degradable blocks in the whole pectin sample as described in detail previously (Guillotin et al., 2005).

The degree of blockiness of saponified amidated pectins (O5sap-O27sap) was determined (Table I) to analyse the distribution of amide groups. When the degree of amidation was decreasing (DAm 27→5%) the degree of blockiness of amide groups was increasing (DB 11.4-14.8%) as a result of the higher amount of free GalA present. The same conclusions can be made when analysing the DB_{abs}.

The saponified amidated pectin O16sap was compared to a similar DS pectin (DM17). For this methyl-esterified pectin, Daas et al. calculated a DB_{abs} of 38.9% (Daas et al., 2001), which is much higher than the DB_{abs} found in our study with pectin O16sap (DB_{abs} of 10.4%). This indicated that the amide groups were semi-randomly distributed over the pectic backbone as it has been observed previously (Anger & Dongowski, 1988; Voragen, Schols, Clement & Pilnik, 1984). These results were in contrast with previous findings where a blockwise distribution of the amide groups was suggested (Racape et al., 1989).

It was proven already that commercial HM pectins were constituted of several pectic populations (Guillotin et al., 2005; Kravtchenko, Berth, Voragen & Pilnik, 1992; Kravtchenko, Voragen & Pilnik, 1992b; Ralet, Bonnin & Thibault, 2001; Ralet & Thibault, 2002; Schols, Reitsma, Voragen & Pilnik, 1989). Therefore, different pectic populations were expected to be present for amidated pectins as well. During the heterogeneous amidation process in the presence of a mixture of water/alcohol/ammonia (Anger & Dongowski, 1988), pectins are not soluble. It was suggested (Racape et al., 1989) that only the outer layers of pectin particles are in contact with the solvent and available for alkali attack. This would explain the blockwise distribution of the amide groups. The amidated pectins analysed in our study were also non-homogenous polymers. Only few PG degradable blocks were indicated

in these populations. Therefore, these amidated pectins could not be qualified as fully blockwise amidated pectins. It is important to note that the distribution of the amide groups will vary according to the amidation process: the distribution of these substituents in pectins amidated in a homogeneous phase (concentrated ammonia (Black & Smit, 1972)) is expected to be different than the distribution of the pectins amidated in a heterogeneous phase (Anger & Dongowski, 1988).

3.3.3. Distribution of both amide groups and methyl-esters

Three LMA pectins (O16, O20 and O27) were prepared from the same pectin preparation, which meant that the initial distribution of the methyl-esters was the same for all three samples. These pectins contained a similar degree of substitution (respectively, 52, 49 and 51%) but a different ratio of amide groups versus methyl-esters (respectively, ratio amide groups/methyl-esters of 0.4, 0.7 and 1.35). They were analysed to check whether a different ratio of amide groups would change the degree of blockiness of the overall substitutents (table I). These three pectins had a similar DB (9.2-10%) and DB_{abs} (4.4-4.9%) suggesting a similar distribution of the substituents. This similar distribution might be due to the same mother pectin prior to amidation. Methyl-esters were only replaced by amide groups resulting in a similar distribution of the substituents. These results also emphasized on the similar behavior of the endo-PG towards amide groups and methyl-esters as observed previously.
The LMA pectins O16-O27 were compared to a methyl-esterified pectin with a similar degree of substitution (DM56%) studied previously (Daas et al., 1999). Daas et al. found a DB_{abs} value of 13.9% for a blockwise DM56 pectin, and a DB_{abs} of 1.7% for a random DM52 pectin (Daas et al., 1999): the DB_{abs} values of the three LMA pectins (DB_{abs} from 8 to 10%) were in between a random and a blockwise distribution of the substituents and thus semi-random.

3.3.4. Comparison of the distribution of the substituents of two LMA pectins with similar chemical characteristics but different physical properties

Two commercial LMA pectins (D and G) with similar GalA, DM and DAm were analysed since they presented totally different gelling properties in the presence of calcium (pectin D was more calcium sensitive compared to pectin G). To check whether these physical differences were due to a different distribution of the methyl-esters and/or amide groups, these two samples have been analysed in more detail. The DB_{abs} of pectin D was slightly

lower compared to pectin G but the endo-PG degradable blocks were similar when the pectins were saponified (comparison of pectin D with Dsap and G with Gsap; Table I). It seems that the removal of methyl-esters did not result in the production of more unsubstituted free GalA blocks large enough to be degraded by endo-PG. This would indicate that the methyl-esters were rather regularly distributed. The ratio of substituted oligomers versus non-substituted oligomers (S^+/S^- ratio) was determined to get information about the position of the blocks. A high S^+/S^- ratio is indicative of closed or longer PG degradable blocks over the pectic backbone. The free GalA blocks of pectin G were found to be more closely neighboring or longer compared to pectin D (respectively, S^+/S^- ratio of 1.4 and 1.9). It may be that the higher calcium sensitivity of pectin D is due to the distribution of the free GalA blocks.

4. Conclusions

A method has now been validated to determine the degree of blockiness of amide groups from LMA pectins by using the endo-polygalacturonase from K*luyveromyces fragilis*. The enzyme seems to have the same specificity towards methyl-esters and amide groups. All oligomers released by the enzyme and analysed on HPAEC pH 5 with sodium hydroxide post column addition were characterized. The distribution of amide groups over the pectic backbone of the LMA pectins analysed in this study appeared to be semi-random while the distribution of the methyl-esters was regular for some LMA pectins (only few PG degradable blocks). Obviously, the distribution of both methyl-esters and amide groups depended on the distribution of the starting material and might cause a wide range of blockiness for amidated pectins. The DB values of two commercial LMA pectins with different gelling behavior in the presence of calcium but with similar chemical characteristics were analysed and the distribution of the substituents of the pectin D seemed to be slightly more random compared to pectin G. However, the DB values obtained corresponded to an average and it was shown that these pectins may contained different pectic populations with different features as observed previously for HM pectins (Guillotin et al., 2005). Differences in populations and in their characteristics may further explain the different physical properties of these LMA pectins.

References

Alebeek G.-J. W. M. v., Schols H. A., Voragen A. G. J. (2001). Amidation of methyl-esterified oligogalacturonides: examination of the reaction products using MALDI-TOF MS. *Carbohydrate Polymers*, *46*, 311-321.

Anger H., Dongowski G. (1988). Amidated pectins - characterization and enzymatic degradation. *Food Hydrocolloid.*, *2*, (5), 371-379.

Barrett A. J. B., Northcote D. H. (1965). Apple fruit pectic substances. *Biochemical Journal*, (94), 617-627.

Black S. A., Smit C. J. B. (1972). The effect of demethylation procedures on the quality of low-ester pectins used in dessert gels. *Journal of Food Science*, *37*, (II), 730-732.

Christensen P. E. (1954). Methods of grading pectin in relation to the molecular weight (intrinsec viscosity) of pectin. *Food Research*, *19*, 163.

Daas P. J. H., Arisz P. W., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1998). Analysis of partially methyl-esterified galacturonic acid oligomers by high-performance anion-exchange chromatography and matrix-assisted laser desorption/ionization time-of flight spectrometry. *Analytical Biochemistry*, *257*, 195-202.

Daas P. J. H., Boxma B., Hopman A. M. C. P., Voragen A. G. J., Schols H. A. (2001). Nonesterified galacturonic acid sequence homology of pectins. *Biopolymers*, *58*, 1-8.

Daas P. J. H., Meyer-Hansen K., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research*, *318*, 135-145.

Daas P. J. H., Voragen A. G. J., Schols H. A. (2000). Characterisation of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research*, *326*, 120-129.

Darvill A. G., McNeill M., Albersheim P. (1978). Structure of plant cell walls: VIII. A new pectic polysaccharide. *Plant Physiology*, 62, 418-422.

De Vries J. A., Voragen A. G. J., Rombouts F. M., Pilnik W. (1981). Extraction and purification of pectins from alcohol insoluble solids from ripe and unripe apples. *Carbohydrate Polymers*, *1*, 117-127.

Food Chemical Codex, (1981) 3rd Ed., National Academy of Sciences, Washington, DC

Guillotin S. E., Bakx E. J., Boulenguer P., Mazoyer J., Schols H. A., Voragen A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*, 391-398.

Huisman M. M. H., Oosterveld A., Schols H. A. (2004). New method for fast determination of the degree of methylation of pectins by headspace GC. *Food Hydrocolloids.*, *18*, (4), 665-668.

Kravtchenko T. P., Berth G., Voragen A. G. J., Pilnik W. (1992). Studies on the intermolecular distribution of industrial pectins by means of preparative size exclusion chromatography. *Carbohydrate Polymers*, *18*, 253-263.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992a). Analytical comparison of three industrial pectin preparations. *Carbohydrate Polymers*, *18*, 17-25.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992b). Studies on the intermolecular distribution of industrial pectins by means of preparative ion-exchange chromatography. *Carbohydrate Polymers*, *19*, 115-124.

Lecacheux D., Brigand G. (1988). Preparative fractionation of natural polysaccharides by size exclusion chromatography. *Carbohydrate Polymers*, *8*, 119-130.

Lofgren C., Guillotin S., Evenbratt H., Schols H., Hermansson A.-M. (2005). Effects of calcium, pH and blockiness on kinetic rheological behavior and microstructure. *Biomacromolecules*, *6*, 646-652.

Lootens D., Capel F., Durand D., Nicolai T., Boulenguer P., Langendorff V. (2003). Influence of pH, Ca concentration, temperature and amidation on the gelation of low methoxyl pectin. *Food Hydrocolloids*, *17*, 237-244.

McNeill M., Darvill A. G., Albersheim P. (1980). Structure of plant cell walls: X. Rhamnogalacturonan I A structurally complex polysaccharide in the walls of suspension-cultured sycamore cells. *Plant Physiology*, 66, 1128-1134.

Neukom H., Amado R., Pfister M. (1980). Neuere erkenntnisse auf dem gebiete der pektinstoffe. *Lebensm.wiss.u technology*, 13, 1-6.

Owens H. S., Svenson H. A., Schultz T. H., (1933). In: *Natural Plant Hydrocolloids, Advances in Chemistry*; American Chemical Society, 10.

Pasculli R., Geraeds C., Voragen F., Pilnik W. (1991). Characterization of polygalacturonases form yeast and fungi. *Lebensmittel Wissenschaft und Technologie*, *24*, 63-70.

Pilnik W., Voragen A. G. J., (1991). In: *Food Enzymology*; Fox P. F., London, Elsevier Applied Science, 303-306.

Powell D. A., Morris E. R., Gidley M. J., Rees D. A. (1982). Conformations and interactions of pectins II. Influence of residue sequence on chain association in calcium pectate gels. *Journal of molecular biology*, 155, 517-531.

Racape E., Thibault J. F., Reitsma J. C. E., Pilnik W. (1989). Properties of amidated pectins II. Polyelectrolyte behavior and calcium binding of amidate pectins and amidated pectic acids. *Biopolymers*, *28*, 1435-1448.

Ralet M. C., Bonnin E., Thibault J.-F. (2001). Chromatographic study of highly methoxylated lime pectins de-esterified by different pectin methyl-esterases. *Journal of Chromatography B*, *753*, 157-166.

Ralet M. C., Thibault J.-F. (2002). Interchain heterogeneity of enzymatically deesterified lime pectins. *Biomacromolecules*, *3*, 917-925.

Rolin C., (2002). Commercial pectin preparations. In: *Pectins and their Manipulation*; Seymour G. B., Knox J. P., Blackwell Publishing Ltd., 222-239.

Schols H. A., Reitsma J. C. E., Voragen A. G. J., Pilnik W. (1989). High-performance ion exchange chromatography of pectins. *Food Hydrocolloids*, *3*, (2), 115-121.

Thibault J.-F., Rinaudo M. (1986). Chain association of pectic molecules during calcium-induced gelation. *Biopolymers*, *25*, 456-468.

Van Deventer-Schriemer W. H., Pilnik W. (1987). Studies on pectin degradation. *Acta Alimentaria*, *16*, 143.

Voragen A. G. J., Pilnik W., Thibault J.-F., Axelos M. A. V., Renard C. M. G. C., (1995). Pectins. In: *Food polysaccharides and their applications*; Stephen A. M., New York, Marcel Dekker Inc., 287-339.

Voragen A. G. J., Schols H. A., Clement A. J. J., Pilnik W., (1984). Enzymic analysis of pectins. In: *Gums and stabilisers for the food industry 2. Applications of Hydrocolloids*; Philips G. O., Wedlock D. J., Williams P. A., ed., Elsevier London, 517-521.

Chapter 6

Chromatographic and enzymatic strategies to reveal differences between amidated pectins on molecular level.

To be submitted in Biomacromolecules as:

S.E Guillotin, N. Mey, E. Ananta, P.Boulenguer, H.A. Schols and A. G. J. Voragen.

Abstract

When applying pectins as a food ingredient, the "routine' analysis usually performed (galacturonic and neutral sugar content, molecular weight distribution and level of methyl-esterification and amidation) does not always explain differences between pectins having different functional properties. This is particularly true for low methyl-esterified amidated pectins (LMA) since not much is known so far on the two 'independent' and complex distributions of both methyl esters and amide groups. To get more knowledge about the chemical structure of such pectins, the distribution of amide groups within two commercial LMA pectins was studied after removal of the methyl-esters followed by fractionation of the different populations by anion exchange chromatography. Despite the different elution behavior on the anion exchange column, the different populations had almost equal degrees of amidation suggesting different distributions of the amide groups. This was indeed substantiated by establishing the degree of blockiness (DB) by using endo-polygalacturonase as an analytical tool. However, the distribution of amide groups for most populations should be considered as semi-random since blockwise distributed pectins would have much higher DB values. Digestion of populations obtained after anion exchange chromatography of the methyl esterified amidated pectins 'as is' showed a rather random distribution for almost all populations. However, a striking difference between the different populations was that, despite of the same level of substitution, the ratio between amide groups and methyl esters varied significantly indicating an heterogeneous amidation process.

1. Introduction

Nowadays, pectin is widely used as gelling and thickening compound, but is also known for its health effect such as antidiarrhea and detoxicant properties, the regulation and protection of gastrointestinal tract and anti-tumour activity (Voragen, Pilnik, Thibault, Axelos & Renard, 1995; Waldron & Selvendran, 1993). It has been demonstrated that pectin is a complex polysaccharide composed of an α-1,4-linked D-galacturonic acid (GalA) backbone (smooth regions). This homogalacturonan is interrupted by alternating rhamnose/GalA sequences where neutral sugars are substituted to the rhamnose moieties (hairy regions) (Barrett & Northcote, 1965; Darvill, McNeill & Albersheim, 1978; De Vries, den Uyl, Voragen, Rombouts & Pilnik, 1983; De Vries, Rombouts, Voragen & Pilnik, 1982; De Vries, Rombouts, Voragen & Pilnik, 1983; De Vries, Voragen, Rombouts & Pilnik, 1981; McNeil, Darvill & Albersheim, 1980; Neukom, Amado & Pfister, 1980). Commercial pectins are extracted from lemon peels or apple pommace mainly yielding high methyl-esterified (HM) pectins meaning that 50% or more of the galacturonic acids are methyl-esterified. These HM pectins can be de-esterified to produce low methyl-esterified (LM) pectins (less than 50% of the galacturonic acid residus in the backbone is methyl-esterified). Both types of pectins have completely different gelling conditions. The LM pectins are used mainly in the presence of calcium at neutral pH but as well at acidic conditions without calcium (Gilsenan, Richardson & Morris, 2000; Voragen et al., 1995). HM pectins are used at low pH (below 3.5) in the presence of sugar and without calcium addition (Voragen et al., 1995). The gels of LM pectins are known to be thermoreversible, which is not the case for the HM pectin gels (Rolin & De Vries, 1990). A third category of pectin is obtained by chemical amidation of HM pectins to obtain low methyl-esterified amidated pectins (LMA pectins). These LMA pectins need less calcium to gel and are claimed to be perfectly thermoreversible (Racape, Thibault, Reitsma & Pilnik, 1989). Furthermore, the firmness and the strength of the gels obtained in the presence of calcium are higher for LMA compared to the LM pectins with similar degree of substitution (Black & Smit, 1972).

The gelling mechanism of amidated pectins is not completely understood yet. It seems that both the egg-box mechanism described previously for LM pectins (Voragen et al., 1995) and the stabilization of the junction zones with the hydrogen bonds of amide groups on pectins (Alonso-Mougan, Meijide, Jover, Rodriguez-Nunez & Vazquez-Tato, 2002) play an important role. The gelling mechanisms of pectins are influenced by several factors such as

their molecular weight (Christensen, 1954; Owens, Svenson & Schultz, 1933; Van Deventer-Schriemer & Pilnik, 1987), their total charge and the distribution of their charges over the pectic backbone (Lofgren, Guillotin, Evenbratt, Schols & Hermansson, 2005; Rolin & De Vries, 1990; Thibault & Rinaudo, 1986; Voragen et al., 1995).

In this study, we set out for a more detailed characterisation by using anion exchange chromatography of two LMA pectins with similar chemical characteristics but different gelling behavior in the presence of calcium. Pectins and pectic fractions were studied in the original form and also after saponification to study the distribution of amide groups only. Populations were also digested with endo-polygalacturonase to determine the distribution of the substituents over the galacturonan backbone.

2. Material and methods

2.1. Pectin samples

The samples D and G were kindly provided by Degussa Texturant Systems. The galacturonic acid content (GalA), the degree of methyl-esterification and the degree of amidation of these pectins are described in Table I.

2.2. Saponification of pectins D and G

Pectin samples were wetted with ethanol, solubilised in water (0.8%) and cooled on ice. Then an equal volume of NaOH (0.1 M) was added. The solutions were stirred and stored overnight at 4°C. An equal volume of acetic acid (0.1 M) was added to neutralise. Acetate and methanol were removed by dialysis with dialysis tubing (cut off 12 – 14 kDa for proteins) and samples were freeze-dried. No β-elimination occurred as indicated by HPSEC analysis of the saponified pectins (results not shown).

2.3. Preparative chromatography of commercial pectins

An Akta explorer system was used for separation of pectins on a preparative scale. Pectin (0.5 g) was dissolved in 100 ml of 0.03 M of sodium phosphate buffer. Elution was performed on a Source-Q column (115 × 60 mm; Amersham Biosciences) using “Millipore” water during 4 column volumes (CV) followed by a linear gradient in steps: 0 to 0.12 M of sodium phosphate buffer (pH 6) in 13 CV at 60 ml/min; 0.12 M to 0.42 M of sodium phosphate buffer

(pH 6) in 44 CV; 0.42 M to 0.6 M sodium phosphate (pH 6) in 2 CV and finally 8.5 CV of 0.6 M sodium phosphate pH 6. The column was washed with 1 M sodium hydroxide for 5 CV. Detection was accomplished with an UV detector set at 215 nm.

The fractions (250 ml) were pooled and ultrafiltrated with a Pellicon 10 kDa membrane (size of 50 cm^2) till a conductivity < 10 μS. After ultrafiltration, the fractions were freeze-dried. Then the different pools were resuspended and dialysed with dialysis tubing (cut of 12-14 kDa for proteins) against "Millipore water" to remove last traces of salts prior to freeze-drying.

2.4. Uronic acid content

Pectin solutions (60 μg/ml) were boiled (1h), cooled and then saponified with sodium hydroxide (40 mM). The uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl method (Ahmed & Labavitch, 1977; Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979).

2.5. Neutral sugar content

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984) using inositol as an internal standard. The samples were treated with 72% (w/w) H_2SO_4 (1h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C and the constituent sugars released were analysed as their alditol acetates.

2.6. Methyl-ester content

The methyl-ester content was determined by GC headspace analysis of the free methanol released after alkaline de-esterification of pectins (Huisman, Oosterveld & Schols, 2004).

2.7. Digestion of the pectins with endo-polygalacturonase to determine the degree of blockiness of the free GalA

Samples (5mg/ml) were diluted in sodium acetate 50 mM pH 5 and incubated with an overdose of endo-polygalacturonase of *Kluyveromyces fragilis* (0.04 units/ml) for 24 hours. The specific activity of this enzyme for PGA was 128 U/mg. Pectin digests were prepared by incubation of pectic solutions with endo-polygalacturonase (0.04 units/ml) for 24 hours. As a

result of the extended endo-polygalacturonase incubation employed, only end-products were observed as was demonstrated by the use of an excess of enzymes and longer incubation times. Oligomers released were analysed by HPAEC or CE as described below and the degree of blockiness was calculated. The degree of blockiness (DB) is the amount of mono-, di- and trigalacturonic acid released by the endo-polygalacturonase related to the amount of free GalA present in the sample. The absolute degree of blockiness (DB_{abs}) is the amount of mono-di- and trigalacturonic acid released by the endo-polygalacturonase related to the total amount of GalA (free and substituted GalA) present in the sample (Guillotin, Bakx et al., 2005).

2.8. CE analysis to determine the degree of amidation, degree of substitution and degree of blockiness of amidated pectins

Analysis of the degree of amidation was performed as described previously (Guillotin, Ananta et al., 2005). Phosphate buffer 50 mM pH 7 was used as electrophoresis buffer. Samples and standards were wetted in 10 µl ethanol and dissolved in the phosphate buffer (5 mg/ml). Experiments were carried out on an automated CE system (P/ACE MDQ) equipped with an UV Detector (stated at 190 nm and 200 nm). A fused silica capillary internal diameter 50 µm, total length of 50.2 cm with 40 cm length capillary from inlet to detector was used and thermostatted at 25^{o}C. New capillaries were conditioned by rinsing for 15 min with 0.1 M NaOH, 30 min distilled water and 30 min phosphate buffer. Between two runs the capillary was washed for 2 min with 0.1 M NaOH, 1min with distilled water and 2 min with phosphate buffer. All solutions were filtered on a 0.2 µm membrane. Samples (50 µl) were loaded hydrodynamically (5 sec at 9.5 Psi) and electrophoresis was performed across a potential difference of 20 kV (during 37 min in phosphate buffer pH 7) for DS, DM and DAm analysis and 17 kV for the DB analysis (performed only on the populations fractionated from the crude pectins D and G). The separation process is performed in normal polarity.

The shift of the electro-osmotic flow (eof), observed sometimes within a sample sequence, was corrected by using the following transformation: $t_{cor} = 1/ [(1/t) –(x)]$ where t_{cor} is the migration time of the sample corrected from the eof shift, t is the migration time of the sample observed, x is the value to match the eof migration time for all samples.

The correlation of the Electrophoretic Mobility (EM) with total charge expected was used for determination of the degree of amidation. The equation to calculate the EM is described below

$EM = EM_p - EM_m = (lL /V) [(1/t_p) – (1/t_m)]$

where EM_p corresponds to the observed mobility of the pectin and EM_m to the observed mobility of the eof, l is the distance from the inlet to the detector, L is the total length of the capillary, V the applied voltage, t_p and t_m are the migration times of pectins and neutral markers, respectively (Zhong, Williams, Goodall & Hansen, 1998).

2.8.1. HPAEC pH5 analysis of oligomers for the determination of the degree of blockiness

Oligomers released in endo-polygalacturonase digests (of the populations fractionated from Dsap and Gsap) were analysed by HPAEC on a Thermo-Quest HPLC system (100 µl injection) equipped with a Dionex CarboPac PA1 anion exchange column (250 × 2mm) and a CarboPac PA1 pre-column (50 × 2mm). The column was equilibrated with 0.01 M sodium actetate pH 5 during 10 min. Elution was performed in two steps: a linear gradient from 0.01 to 0.55 M of sodium acetate pH 5 in 40 min and another linear gradient from 0.55 M to 1 M sodium acetate pH 5 in 60 min with a flow of 0.2 ml/min. The gradient was hold at 1 M sodium acetate pH 5 for 10 min. The PAD detector (Dionex) was equipped with a gold working electrode and an Ag/AgCl reference electrode. Detection of the oligomers was possible after post column sodium hydroxide addition (1 M; 0.2 ml/min). Mono-, di- and tri-GalA peaks were integrated by using the peakfit software (Aspire Software International).

3. Results and discussion:

3.1. Separation of pectic populations from saponified LMA pectins by preparative anion exchange chromatography

Two LMA pectins were analysed to understand their different gelling behavior in presence of calcium. Pectin D was found to be more sensitive to calcium compared to pectin G during gel formation (results not shown), but routine chemical analysis (GalA and NS content, DM and DAm; Table I) showed similar chemical characteristics. The degree of blockiness (DB), which is a parameter to reveal the distribution of the charges over the pectic backbone, has been introduced previously (Daas, Meyer-Hansen, Schols, De Ruiter & Voragen, 1999; Daas, Voragen & Schols, 2000; Guillotin, Bakx et al., 2005). Pectins are digested with an endo-polygalacturonase known to release mono-, di and triGalA oligomers when sequences of more than four free GalA blocks are present. The DB is the percentage of these non-methyl-

esterified GalA oligomers liberated by the endo-PG related to the total number of non-methyl-esterified GalA present in the pectin (Daas et al., 1999; Daas et al., 2000; Guillotin, Bakx et al., 2005).

From previous results it was suggested that amide groups and methyl-esters had the same effect on endo-PG action when pectins were digested (Anger & Dongowski, 1988; Guillotin, Schols, van Kampen, Boulenguer & Voragen, 2005). Using the amount of mono-, di- and triGalA released by the endo-PG, the DB of the amidated pectins was determined. Since the DB of pectins D and G was found to be similar (9% and 10%, respectively), this did not explain their different gelling behaviour.

Table I: Characteristics of crude and saponified LMA pectins D and G as well as the populations obtained after fractionation on a preparative Source-Q column of the saponified LMA pectins Dsap and Gsap.

Samples	GalA (w/w%)	NS (w/w%)	DAm[a] (%)	DB[b] (%)	DB_{abs} (%)
D	68	5	19 (DM29)	9	7
Dsap	71	5	19	14	11
$D1_s$	7	16			
$D2_s$	65	3	22	11	9
$D3_s$	70	2	20	14	11
$D4_s$	69	2	15	23	19
G	70	5	18 (DM31)	10	6
Gsap	69	5	18	16	13
$G1_s$	14	5			
$G2a_s$	22	4	24	11	9
$G2b_s$	35	2	20	15	12
$G2c_s$	61	2	17	14	12
$G3_s$	68	2	16	9	8
$G4_s$	65	2	16	17	14
$G5_s$	4	1	19	34	28

[a] DAm determined using CE method
[b] DB determined with HPAEC method

The distribution of the substituents of these pectins were rather random since Daas et al. found a higher DB (33% for a DM 56.4 pectin) for a blockwise methyl-esterified pectin with a similar DS (Daas, Boxma, Hopman, Voragen & Schols, 2001) as the amidated pectins.

Recently, commercial HM pectin preparations were found to be composed of populations with different characteristics concerning the total charge and the distribution of these charges (Guillotin, Bakx et al., 2005), which may account for the different gelling behavior of the pectins. The amidated pectin preparations were suspected to contain different pectin populations with different chemical features as well, therefore the pectic populations of amidated pectins were separated on preparative anion exchange chromatography by using the same approach as described previously (Guillotin, Bakx et al., 2005).

Since LMA pectins contain both methyl-esters and amide groups, we saponified pectins to focus first on the distribution of the amide groups. The DB of saponified pectin G (Gsap, DB 16%; Table I) was slightly higher than the one of saponified pectin D (Dsap, DB 14%) indicating a slightly more blockwise distribution of the amide groups in pectin G compared to pectin D. However, these rather low DB values indicated a rather random distribution of the amide groups compared to a blockwise methyl-esterified pectin (DB of 39% for a DM 17 pectin) with similar DS (Daas et al., 2001). From the slightly different DB results of the saponified amidated pectins and the similar DB of the crude amidated pectins, methyl-esters of the crude pectin G were suggested to be more randomly distributed compared to pectin D.

As expected, several pectic populations were also found to be present in saponified amidated pectins Dsap and Gsap after separation on anion exchange chromatography (Figure 1).

The elution profiles of saponified pectins Dsap and Gsap were rather similar with only differences in the relative proportion of the populations present (fractions 40-69, 70-77 and 78-83, respectively). Pectin Gsap contained slightly more pectin molecules eluting at high ionic strength and less pectin molecules eluting at lower ionic strength compared to pectin Dsap. Neutral sugars were found in populations eluting at low ionic strength (mainly $D1_s$), but the NS content was low (results not shown).

The populations may differ in their total charge and/or in the distribution of the charges since it has been demonstrated that the elution behavior of pectins on this column is sensitive to these two different features (Guillotin, Bakx et al., 2005). Fractions were collected and pooled as shown in Figure 1 and characterised (Table I). The recovery of GalA content was 89% and 91% for pectins Dsap and Gsap, respectively.

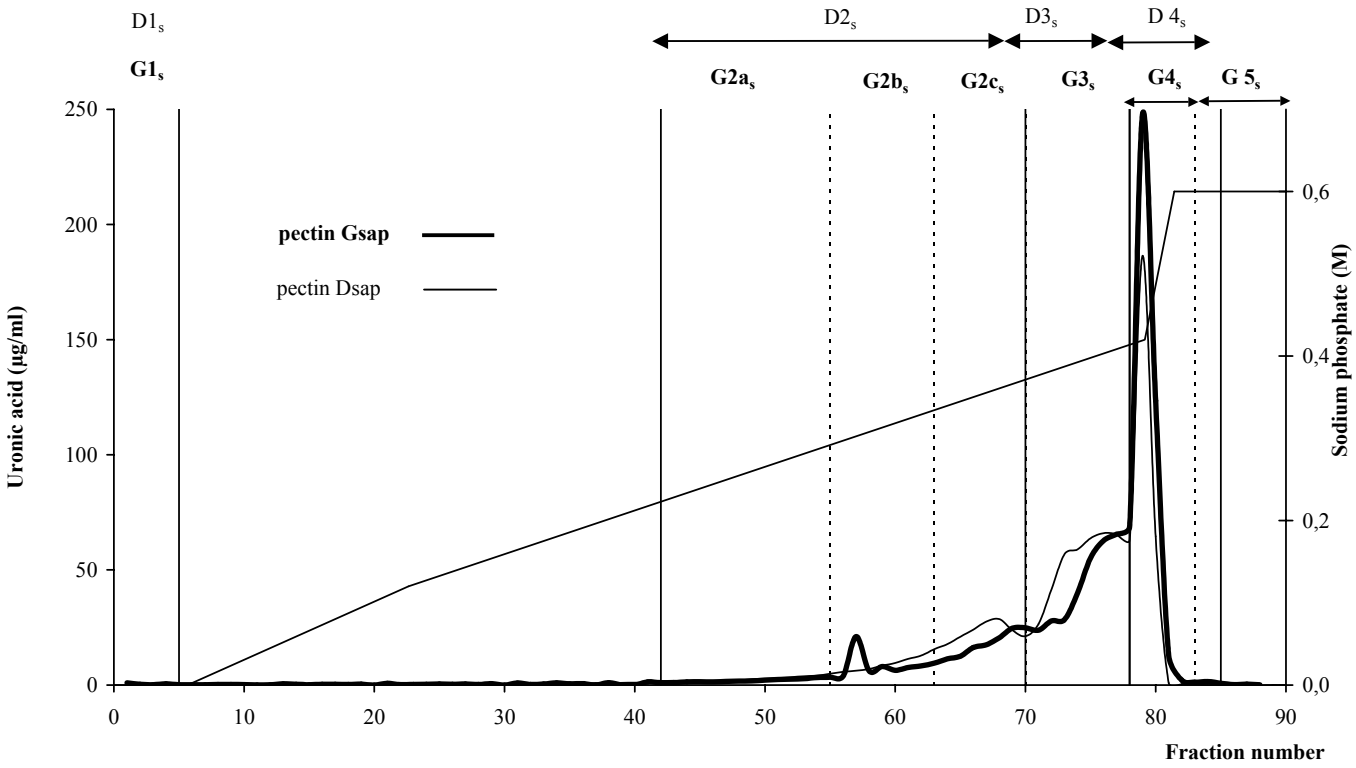

Figure 1: Preparative anion exchange chromatography of saponified pectins Dsap and Gsap on a Source-Q column. The elution profiles were obtained after determination of the uronic acid content in each fraction. The fractions (250 ml) were pooled as indicated.

The GalA content was low for populations eluting at low ionic strength ($D1_s$, $G1_s$, $G2a_s$ and $G2b_s$) and high ionic strength ($G5_s$) as it has been observed previously for methyl-esterified pectins (Kravtchenko, Voragen & Pilnik, 1992b). These populations were not investigated further since they represented less than 5% of the total GalA present in the crude pectin. The NS content was low for both commercial pectins as a result of the acid extraction in the manufacturing process (Guillotin, Bakx et al., 2005; Kravtchenko, Voragen & Pilnik, 1992a). The other populations had higher GalA contents: 61 to 70% in w/w. The degree of amidation as measured by CE (Guillotin, Ananta et al., 2005) decreased for populations eluted at high ionic strength of the eluting buffer: from 22-15% for $D2_s$-$D4_s$ and from 24-16% for $G2a_s$-$G4_s$. The pectin fraction $G5_s$ was deviating from this rule since the DAm was slightly higher compared to the DAm of pectin $G4_s$ (19% and 16%, respectively). The area of each population as defined in Figure 1, was integrated by using the software "Peakfit" to calculate the recovery

of amide groups. The recovery of amide groups was 100% for Dsap populations and 84% for Gsap populations. The elution behavior of the populations on the anion exchanger could not be explained by the DAm only since populations $G3_s$ and $G4_s$ for example had the same DAm whereas they eluted at different ionic strength. The parameter reflecting the distribution of free and amidated carboxyl groups (degree of blockiness) was determined for the relevant populations. We saw previously that population $D2_s$, $D3_s$ and $D4_s$ eluted as a function of their charge. In addition, $D4_s$ was found to have a more blockwise distribution of the amide groups compared to $D2_s$ and $D3_s$. An increase of PG degradable blocks was also observed for populations $G3_s$, $G4_s$ and $G5_s$ (DB of 10%, 18% and 34%, respectively). The populations $G3_s$ and $G4_s$ presented the same DAm but $G4_s$ had a more blockwise distribution of amide groups explaining the later elution of this population. The late elution time of population $G5_s$ also had to be attributed to a considerably more blockwise distribution of the free GalA (DB of 34%) since the DAm was even higher compared to populations $G2c_s$, $G3_s$ and $G4_s$.

Recently we also introduced the DB_{abs} corresponding to the ratio of GalA residues released from endo-polygalacturonase and the total number of GalA residues (substituted and non substituted ones) in the pectic population. The Source-Q column was found to discriminate between pectic populations with different DB_{abs} (Guillotin, Bakx et al., 2005). The more endo-PG degradable blocks in the pectic populations, the later the elution on the anion exchanger. This was observed as well in this study except for the population $G3_s$.

When we compared the characteristics of Dsap and Gsap populations, we observed that even though populations eluted at the same ionic strength, they slightly differed either in their DAm or in the distribution of the amide groups. For example, the elution of population $D3_s$ $G3_s$ was dictated by the DAm and not by the difference in the distribution of the amide (DB_{abs} of 11% and 8%, respectively).

Pectic populations were found to be different with respect to the level and distribution of the amide groups. To obtain more information about the crude pectins D and G, their pectic populations were isolated by using anion exchange chromatography and characterised.

3.2. Separation of pectic populations from crude LMA pectins on preparative anion exchange chromatography

Pectins D and G were fractionated by preparative anion exchange chromatography (Figure 2). Obviously, pectic populations from pectins D and G eluted earlier compared to those of pectin

Dsap and Gsap as a result of the higher degree of substitution (DS of 46% and 50% compared to 18 and 19%, respectively) and consequently lower netto charge. Both crude pectins showed quite similar elution profiles, but pectin D contained more pectin molecules eluting at lowest ionic strength (from 0.2 M-0.25 M phosphate buffer) and at high ionic strength (0.4 M phosphate buffer) compared to pectin G. Fractions were pooled for both pectins D and G as shown in figure 2, and characterised (Table II).

The GalA recovery was 83% for both pectins D and G. The populations eluting at low ionic strength (D1, G1, D2 and G2) had a lower GalA content (respectively, 4%, 10%, 48% and 38% in w/w) while the GalA content of the other pectin fractions was in a higher range (57-76% in w/w).

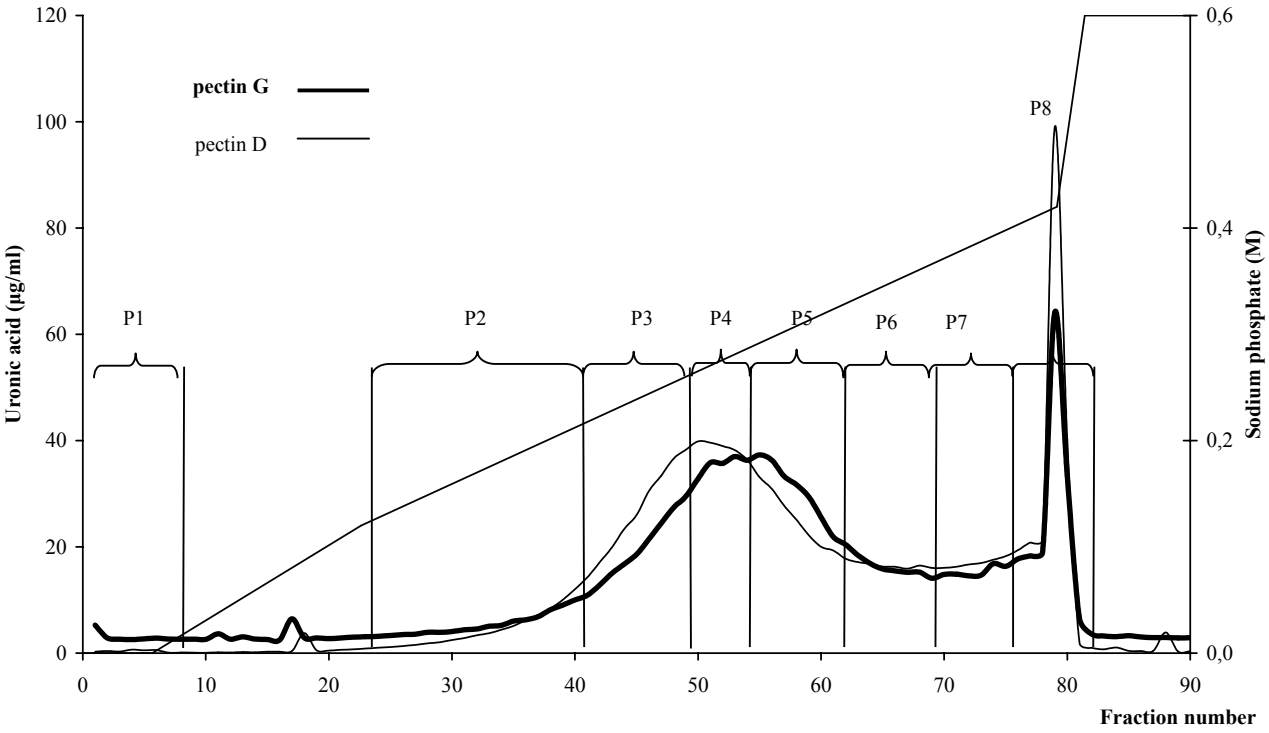

Figure 2: Preparative anion exchange chromatography of pectins D and G on a Source-Q column. The elution profiles were obtained after determination of the uronic acid content in each fraction. The fractions (250 ml) were pooled as indicated.

The NS content was high for the unbound fractions (D1 and G1) and for pectin D2 (50%, 24% and 15% in w/w, respectively) and was lower for the other fractions (2-6% in w/w). The

degree of substitution was different for the pectic populations: 40-22% for D3-D8 and 41-20% for G2-G8 (w/w). The lower the DS, the more binding to the anion exchanger. The DM of the populations was determined by using gas chromatography. The DM was found to decrease when populations were eluted at higher ionic strength (32% for D3 till 3% for D8 and 35% for G3 till 2% for G8). Pectic populations D1 and D2 contained a lower amount of methyl-esters and not enough sample was available to perform DS and DAm determination. The DAm was higher when populations eluted at higher ionic strength (11-20% for D3- D8; and 5-18% for G3-G8). Only pectins D4 and G4 were deviating from this rule (respectively DAm of 8% and 0%).

In general, the more blockwise free GalA are distributed (DB_{abs} from 15-42% for D3-D8 and 15%-46% for G2-G8), the later the elution of the pectins. It is clear that the absolute amount of free GalA blocks (DB_{abs}) was influencing the behavior of the pectic populations on the anion exchanger used and the same observation has been reported previously for the elution of the populations from HM pectins (Guillotin, Bakx et al., 2005).

A striking difference in the characteristics of the populations was the proportion of amide groups and methyl-esters while the DS of the populations was rather similar. For example, pectins G4, G5 and G6 with similar DS (35%, 36% and 34%, respectively) eluted at different ionic strength. Their Am/Me ratio was different: 0, 0.24 and 0.7, respectively (Table II). When the ratio of amide groups versus methyl-esters (Am/Me) was higher the elution of the pectin was later. The same phenomenon was observed for other populations with similar DS such as G7-G8, D6-D7 and D3-D5.

We may speculate that amide groups are stabilising the carboxylate groups resulting in lower pKa values for amidated pectins (McCormick & Elliot, 1986). This would explain stronger interaction of amidated pectins with the anion exchanger compared to methyl-esterified pectins.

A summary of our observations concerning the different parameters (DS, DM, DAm and ratio amide groups versus methyl-esters) of the pectic populations from pectins D and G was given in figure 3. The DS of the populations 3 to 6 was found to be similar to finally decrease for the populations 7 and 8. The DM was decreasing for pectin D while the DAm and the ratio Am/Me was increasing. The same phenomenon was observed for pectin G except for the DM which increased for populations 2 to 4 and then decreased. The DS of populations eluting in the same range of strength were found to be similar. The more calcium sensitivity of pectin D

compared to pectin G may be attributed to a more blockwise distribution of its substituents in some of its populations (D5, D7 and D8).

Table II: Characteristics of the populations of LMA pectins D and G fractionated on a preparative Source-Q column:

Samples	GalA (w/w%)	NS (w/w%)	DS[a] (%)	DM[b] (%)	DAm[c] (%)	Ratio (Am/Me) (%)	DB[d] (%)	DB_{abs} (%)
D	68	4.8	46 ± 0.5	29	19		10	5.4
D1	4	50	nd	15				
D2	48	14.5	nd	42				
D3	73	4.2	40 ± 0.4	32 ± 0.5	8	0.3	25	15
D4	65	4.5	38 ± 0.7	32 ± 0.5	6	0.25	36	22
D5	65	5.3	39 ± 1.6	25 ± 0.5	14	0.7	44	27
D6	62	6.0	34 ± 0.6	18 ± 0.5	16	1	41	27
D7	61	5.7	24 ± 2.5	9 ± 1.0	15	3	52	40
D8	57	3.8	22 ± 1.3	3 ± 0	17	6.6	54	42
G	70	5.2	50 ± 0.1	31	18		11	6.0
G1	10	24.3		2				
G2	38	6.0	41 ± 1	23 ± 0	18	0.8	24	14
G3	63	5.7	38 ± 0.2	35 ± 1.0	3	0.14	35	22
G4	56	3.9	40 ± 0.5	40 ± 0.5	0	0	39	23
G5	75.8	2.0	34 ± 2.6	29 ± 0.5	5	0.24	44	29
G6	57.2	4.7	33 ± 0.2	20 ± 0.5	13	0.7	54	33
G7	59.4	5.2	24 ± 1.9	11 ± 0.5	13	1.2	44	33
G8	64.2	2.7	20 ± 2.6	2 ± 0	18	9	58	46

[a] DS determined with the CE method
[b] DM determined with the GC method
[c] DAm= DS-DM
[d] DB determined with the CE method

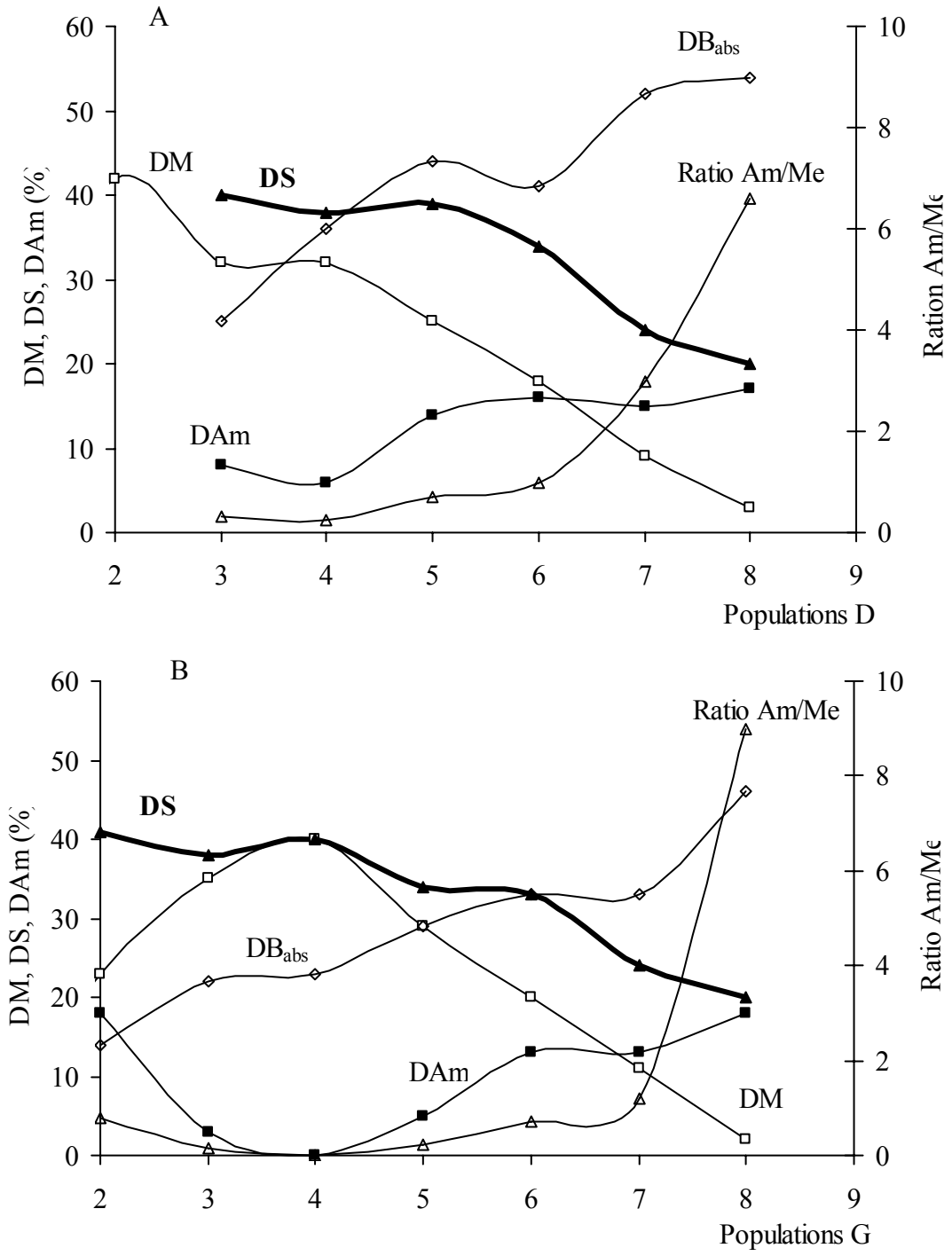

Figure 3: DM, DS, DAm and DB_{abs} of pectic populations from LMA pectins D (A) and G (B).

4. Conclusions

Our study revealed important variations in the features of the pectic populations present in commercial amidated pectins. The results showed differences in the degree of substitution (DS from 20 till 41%), although the populations making up the largest part of the commercial pectin did have rather similar levels of substitution (ca 40). However, the ratio between methyl esters and amide groups changed significantly (0.1 – 9) indicating the presence of pectins almost without methyl esters being present next to molecules rather poor in amide groups. This observation proved the frequently stated suggestion that the amidation process by using a heterogeneous system (insoluble pectins suspended in ethanol) leads to a heterogeneous distribution. However, starting from a HM pectin (DM 70-50) it was surprising that the DS was lowered to the same level (ca 40), despite a different type of substitution. Furthermore, it is striking that the degradability by endo-PG of the different populations of both LMA pectins were rather similar to the degradability of the pectic fractions without methyl esterification, where the amide groups were shown to be distributed not completely random. This would indicate that the methyl esters were rather regularly distributed along the molecule mixed with the amide group distribution in such a way that removal of the methyl esters did not create additional sites for endo-PG.

References

Ahmed A. E. R., Labavitch J. M. (1977). *Journal of Food Biochemistry*, *1*, 361-365.

Alonso-Mougan M., Meijide F., Jover A., Rodriguez-Nunez E., Vazquez-Tato J. (2002). Rheological behaviour of an amide pectin. *Journal of Food Engineering*, *55*, 123-129.

Anger H., Dongowski G. (1988). Amidated pectins - characterization and enzymatic degradation. *Food Hydrocolloids*, *2*, (5), 371-379.

Barrett A. J. B., Northcote D. H. (1965). Apple fruit pectic substances. *Biochemical Journal*, (94), 617-627.

Black S. A., Smit C. J. B. (1972). The effect of demethylation procedures on the quality of low-ester pectins used in dessert gels. *Journal of Food Science*, *37*, (II), 730-732.

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

Christensen P. E. (1954). Methods of grading pectin in relation to the molecular weight (intrinsec viscosity) of pectin. *Food Research*, *19*, 163.

Daas P. J. H., Boxma B., Hopman A. M. C. P., Voragen A. G. J., Schols H. A. (2001). Nonesterified galacturonic acid sequence homology of pectins. *Biopolymers*, *58*, 1-8.

Daas P. J. H., Meyer-Hansen K., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research*, *318*, 135-145.

Daas P. J. H., Voragen A. G. J., Schols H. A. (2000). Characterisation of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research*, *326*, 120-129.

Darvill A. G., McNeill M., Albersheim P. (1978). Structure of plant cell walls: VIII. A new pectic polysaccharide. *Plant Physiology*, 62, 418-422.

De Vries J. A., den Uyl C. H., Voragen A. G. J., Rombouts F. M., Pilnik W. (1983). Structural features in the neutral sugar side chains of apple pectic substances. *Carbohydrate Polymers*, 6, 193-205.

De Vries J. A., Rombouts F. M., Voragen A. G. J., Pilnik W. (1982). Enzymatic degradation of apple pectins. *Carbohydrate Polymers*, 2, 25-33.

De Vries J. A., Rombouts F. M., Voragen A. G. J., Pilnik W. (1983). Distribution of methoxyl groups in apple pectic substances. *Carbohydrate Polymers*, *3*, 245-258.

De Vries J. A., Voragen A. G. J., Rombouts F. M., Pilnik W. (1981). Extraction and purification of pectins from alcohol insoluble solids from ripe and unripe apples. *Carbohydrate Polymers*, *1*, 117-127.

Gilsenan P. M., Richardson R. K., Morris E. R. (2000). Thermally reversible acid-induced gelation of low-methoxy pectin. *Carbohydrate Polymers*, *41*, 339-349.

Guillotin S. E., Ananta E., Bakx E. J., Boulenguer P., Schols H. A., Voragen A. G. J. (to be submitted). Determination of the degree of substitution and degree of amidation of commercial pectins with capillary electrophoresis.

Guillotin S. E., Bakx E. J., Boulenguer P., Mazoyer J., Schols H. A., Voragen A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*, 391-398.

Guillotin S. E., Schols H. A., van Kampen J., Boulenguer P., Voragen A. G. J. (to be submitted). Degree of blockiness of amide groups as indicator for differences between amidated pectins.

Huisman M. M. H., Oosterveld A., Schols H. A. (2004). New method for fast determination of the degree of methylation of pectins by headspace GC. *Food Hydrocolloids*, *18*, (4), 665-668.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992a). Analytical comparison of three industrial pectin preparations. *Carbohydrate Polymers*, *18*, 17-25.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992b). Studies on the intermolecular distribution of industrial pectins by means of preparative ion-exchange chromatography. *Carbohydrate Polymers*, *19*, 115-124.

Lofgren C., Guillotin S., Evenbratt H., Schols H., Hermansson A.-M. (2005). Effects of calcium, pH and blockiness on kinetic rheological behavior and microstructure. *Biomacromolecules*, *6*, 646-652.

McCormick C. L., Elliot D. L. (1986). Water-soluble copolymers. 14. Potentiometric and turbidimetric studies of water-soluble copolymers of acrylamide: comparison of carboxylated and sulfonated copolymers. *Macromolecules*, *19*, 542-547.

McNeil M., Darvill A. G., Albersheim P. (1980). Structure of plant cell walls. X. Rhamnogalacturonan I, a structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. *Plant Physiology*, *66*, 1128-1134.

Neukom H., Amado R., Pfister M. (1980). Neuere erkenntnisse auf dem gebiete der pektinstoffe. *Lebensm.wiss.u technology*, 13, 1-6.

Owens H. S., Svenson H. A., Schultz T. H., (1933). In: *Natural Plant Hydrocolloids, Advances in Chemistry*; Washington DC, American Chemical Society, 10.

Racape E., Thibault J. F., Reitsma J. C. E., Pilnik W. (1989). Properties of amidated pectins II. Polyelectrolyte behavior and calcium binding of amidate pectins and amidated pectic acids. *Biopolymers*, *28*, 1435-1448.

Rolin C., De Vries J. A., (1990). Pectin. In: *Food gels*; Harris, P.J, 401-434.

Thibault J.-F. (1979). Automatisation du dosage des substances pectiques par la méthode au méta-hydroxydiphenyl. *Lebensmittel Wissenschaft und Technologie*, *12*, 247-251.

Thibault J.-F., Rinaudo M. (1986). Chain association of pectic molecules during calcium-induced gelation. *Biopolymers*, *25*, 456-468.

Van Deventer-Schriemer W. H., Pilnik W. (1987). Studies on pectin degradation. *Acta Alimentaria*, *16*, 143.

Voragen A. G. J., Pilnik W., Thibault J.-F., Axelos M. A. V., Renard C. M. G. C., (1995). Pectins. In: *Food polysaccharides and their applications*; Stephen A. M., New York, Marcel Dekker Inc., 287-339.

Waldron K. W., Selvendran R. R., (1993). In Waldron K. W., Johnson I. T., Fenwick G. R. *Food and cancer prevention, chemical and biological aspects* (307-326).: Royal Soc. Chem.

Zhong H. J., Williams M. A. K., Goodall D. M., Hansen M. E. (1998). Capillary electrophoresis studies of pectins. *Carbohydrate Research*, *308*, 1-8.

Chapter 7

Concluding remarks

1. Research motives

Pectins are widely used in the food industry as ingredients for their thickening, gelling and stabilizing properties. Pectin manufacturers are not always able to control the performance of the pectins extracted. One of the reasons is that they do not always have access to the same raw material since the amount and nature of raw material is fluctuating on the world market. As a consequence, pectin industries can not always obtain the best raw material to extract the pectins (e.g. lime or lemon) and they may have to use a different raw material (e.g. orange) which leads to other chemical and physical characteristics of the pectin extracted. Industries can also not control the growing conditions and the harvesting time of the fruits from which their starting material is obtained whereas different maturation stages are known to influence pectin characteristics (De Figueiredo, Lajolo, Alves & Filgueiras, 2002; Fischer, 1993; Redgwell et al., 1997). The same lack in control may take place during the juice extraction process from the fruits and the storage of the peels before the extraction of pectins. All these variations may result in pectins having different molecular weights, different amounts of methyl-esters and different distributions of these constituents over the pectic backbones. A rapid screening of the pectin characteristics allowing a fast prediction of their performance in a specific application (e.g. as a gelling, thickening or emulsifying agent) would be highly beneficial for the pectin producers as well as for the pectin users.

In this study, we therefore focussed on the analysis of the chemical fine structure of commercial pectins. Our goal was to extend our analytical toolbox to analyse the chemical structure in more detail to be able to better understand the different gelling behaviour of pectins with similar chemical features.

A simple and rapid HPLC method was developed to visualise the presence of different pectin populations in commercial pectins, known to have very similar chemical characteristics with "routine" analysis but exhibiting a different gelling behavior. In addition, a preparative chromatography system enabling large-scale fractionation of pectin samples was developed. This allowed us to study pectins in more detail with special emphasis on the distribution of methyl esters over the backbone.

The second part of our research focussed on low methyl esterified amidated (LMA) pectins obtained by chemical amidation of the high methyl-esterified (HM) pectins. Methods were adapted to determine the overall methyl-esterification and amidation in crude pectins as well

as the distribution of both substituents in the pectic populations present in the original pectin mixture.

In this chapter, the chemical characteristics of the different HM and LMA pectic populations will be summarized and possible reasons for these variations such as enzymatic modification in the plant material before extraction of the pectins and/or chemical and enzymatic changes during the process will be discussed. Finally, the relation between the structure of pectins and rheological behaviour will be described.

2. HM pectins

Two HM pectins having similar chemical characteristics (molecular weight, galacturonic acid and neutral sugar content, degree of methyl-esterification) but with different reactivity towards calium ions were analysed. Pectin A was observed to be more calcium sensitive compared to pectin B when they were used as stabilizers in acid dairy drinks (Laurent & Boulenguer, 2003). In our studies (Guillotin et al., 2005), endo-polygalacturonase (endo-PG) from *Kluyveromyces fragiles* was used to analyse the distribution of the non-methyl esterified GalA residues over the pectic backbone. This endo-PG splits within the galacturonan backbone when at least 4 adjacent free GalA are present (Chen & Mort, 1996; Zhan, Jansson & Mort, 1998) releasing mono-, di- and trigalacturonic acids and higher methyl-esterified oligomers which allow us to discriminate between a randomly esterified pectin and a blockwise esterified pectin. The degree of blockiness (DB) corresponds to the number of non-methylesterified residues liberated by endo-PG expressed as the percentage of the total number of non-methylesterified GalA residues in the undigested polymer: the higher the DB, the more blockwise are the free GalA residues distributed over the pectin molecule (Daas, Alebeek, Voragen & Schols, 1999).

The more calcium sensitive pectin A had a blockwise distribution of the free GalA and it is known from literature, that a sequence of 7-20 free GalA residues is required for association with calcium (Braccini, Grasso & Perez, 1999; Kohn, 1975; Powell, Morris, Gidley & Rees, 1982). The higher calcium sensitivity of the pectin A is probably caused by the presence of the non-methyl esterified GalA blocks.

The commercial pectins were fractionated on a Source-Q anion exchanger in order to obtain homogeneous populations and in sufficient amounts for further characterisation. The molecular weight of these pectic populations was found to be rather similar and in general

slightly higher for the pectic populations of pectin A compared to the populations of pectin B. The pectic populations from both pectins A and B showed differences in their degree of methyl-esterification as expected but also differences in the distribution of their substituents. For example, the crude pectin A contained three pectic populations having the same DM but one of these populations has a random distribution of the free GalA blocks (pectin A1) and the two others (pectins A2 and A3) have a more blockwise distribution of the charges (Guillotin et al., 2005).

Taking into account the molecular weight (Mw) of the pectins as shown in Table I and as determined by using HPSEC, the amount of GalA units present in the pectins was calculated (Table I). From these GalA units and the DB_{abs} determined (Guillotin et al., 2005), the amount of free GalA units in PG degradable blocks in the pectin populations per molecule was calculated (GalA-b). Compared to the amount of free GalA present in the pectins A and B, the amount of non-methyl esterified oligomers released by the endo-PG was low in populations A1 and B1 - B3 (3, 2, 6 and 6 free GalA residues, respectively) indicating the presence of short blocks in these populations. Populations A2 - A5 and B4 – B5 had a higher amount of free GalA residues in blocks (12, 13, 26, 117, 69, 88 GalA residues, respectively) and are expected to be more calcium sensitive. These populations with a blockwise distribution of the non-methyl-esterified GalA residues may strongly determined the physical properties of the crude pectins A and B especially for pectin A since these specific pectins are present in higher levels in pectin A. An important conclusion of our research is that only part of the molecules present in a commercial pectin preparation might be responsible for the physical behavior of commercial pectins.

In addition to the digestion of the pectins by an endo-PG to quantify the total amount of free GalA residues in blocks, an exo-polygalacturonase was used to screen for non-methyl-esterified GalA blocks specifically present at the non-reducing end of the pectic polymers (Benen, Vincken & Alebeek, 2002; Korner, Limberg, Christensen, Mikkelsen & Roepstorff, 1999; Limberg et al., 2000). When crude pectins were analysed, the non calcium sensitive HM pectin B had a lower amount of free GalA blocks at the non-reducing end compared to the calcium sensitive pectin A (GalA-nr; Table I) although these “blocks” still were rather short: two GalA residues at the non-reducing end for pectin B compared to five GalA residues for the pectin A. However, the exo-PG digestion was performed on the crude commercial samples resulting in an average value for the number of the free GalA blocks at the non-reducing end for the molecules constituting the pectins. The size of the free GalA blocks at

the non-reducing end was also determined in the populations of pectins A and B and was found to increase with the ionic strength at which the populations were eluted. The size of the free GalA blocks at the non-reducing-end appeared rather short (1-9 free GalA residues) except for the populations B4 containing more extended blocks (28 free GalA residues).
However, it is important to stress that the populations are still slightly heterogeneous (in the molecular weight and charge distribution) therefore it is possible that some polymers within the population present larger blocks at the non-reducing end while the others have shorter GalA blocks at its extremities. However, these results show that the non-methyl-esterified GalA blocks of the pectic populations are hardly long enough for calcium interaction except for population B4.

Table I: Characterisation of two commercial pectins A and B and populations obtained after fractionation on anion exchange chromatography. The galacturonic acid content (GalA), the degree of methyl-esterification (DM), the molecular weight (Mw) and the number of GalA residues in blocks per pectin molecule (GalA-b; GalA-nr; GalA-ir) are presented

samples	GalA (w/w%)	DM (%)	Mw (kDa)	GalA total (units)	Free GalA (units)	GalA-b (units)*	GalA-nr (units)**	GalA-ir (units)***
A	82	74	82	465	121	20	5	15
A1	82	86	90	511	72	3	1	2
A2	59	85	94	534	80	12	3	9
A3	62	86	94	534	75	13	2	11
A4	75	69	98	556	172	26	7	19
A5	57	44	90	511	286	117	9	106
B	74	72	78	443	124	4	2	2
B1	70	92	87	494	40	2	1	1
B2	69	78	87	494	109	6	2	4
B3	75	59	87	494	203	6	4	2
B4	65	64	100	568	204	69	28	41
B5	32	40	74	420	252	88	nd	nd

* GalA-b: free GalA residues in blocks over the GalA backbone
** GalA-nr: free GalA residues in blocks present at the non-reducing-end of the pectin
*** GalA-ir: free GalA residues in blocks present at the reducing-end and/or inside the pectin

The origin of these non-methyl-esterified blocks and the reason for such heterogeneous pectic populations are still unclear and will be discussed later.

2.1. Pectins and pectinases in plant

It is stated in literature that non-methyl-esterified GalA blocks are obtained after degradation of the pectins with plant PME generally assumed to de-esterify pectins in a blockwise way rather than the fungal PME which removes methyl-esters randomly (Ishii, Kiho, Sugiyama & Sugimoto, 1979; Kohn, Furda & Kopec, 1968). The cause of the presence of the wide variation in pectic molecules differing in the level and distribution of the substituents is not known. It is frequently suggested that citrus endogeneous PME may be initiated during the storage of the peel although it is also possible that different molecules are already present in the tissues of the fruits even before juice extraction.

Firstly, a short inventory of the presence of different PME in plants will be shown to discuss the putative modifications of pectins in situ. The genome of members of the citrus plant variety has not been revealed completely yet whereas the entire genome of a well-known plant (*Arabidopsis thaliana*) has been fully characterised. We therefore looked for the *Arabidopsis thaliana* genome to make an inventory of the putative pectinases present in this plant. The characteristics of the pectin methyl-esterases (PME) present in situ may be an indication for the formation of blockwise esterified pectins in situ. However, a gene sequence is not a proof for the expression of the corresponding enzyme neither for the presence of its substrate in the plant tissue.

So far, it is reported in literature that 5 genes coding for PME are present in *Arabidopsis thaliana* and around 60 other putative PME genes are present (Table II). It may be speculated that the PME's genes may be expressed during the growth and the development of the plant tissues. These enzymes can present different mechanism of digestion resulting in pectins with different levels and distribution of the methyl-esters over the pectic backbone. In *Citrus sinensis* (sweet orange), two PME enzymes were expressed (Nairn, Lewandowski & Burns, 1998) and two putative PME enzymes were found so far.

Pectin molecules modified by PME in the cell wall may either change the architecture of the cell walls by making it stronger (formation of calcium gels) or weaker since the free GalA blocks are substrate for the endo-PG and can be degraded.

Table II: Pectin methylesterases in *Arabidopsis thalinana* and *Citrus sinensis* (Family CE 8: carbohydrate Esterase) as found with CAZy (Carbohydrate Active enZymes, http: //afmb.cnrs-mrs.fr/CAZY/GH_28.html; June 2005).

Enzymes	Organism	SwissProt
PME1	*A. thaliana*	Q43867 Q8LA06
PME2	*A. thaliana*	Q42534 Q9SSB0
PME3	*A. thaliana*	O49006 Q93YZ2 Q9LUL7
PME4	*A. thaliana*	O80722 Q8H194 Q9T0P8
PME5	*A. thaliana*	O80721 Q9SMV9
Pectin methylesterase (fragment)	*Citrus sinensis*	O04221
Pectin methylesterase 1.1	*Citrus sinensis*	O04886 O04888
Pectin methylesterase 2.1	*Citrus sinensis*	O04887 O04889
PME4	*Citrus sinensis*	Q8GS16
60 other putative PME genes are present in the *A. thaliana* genome		

The presence of endo-polygalacturonase (endo-PG) in situ which splits between non-esterified GalA residues may indicate that PG degradable blocks are present in the pectins in plants. Therefore we also made an inventory of the polygalacturonases. It was found from different databases that two genes are coding for putative endo-polygalacturonases in *Arabidopsis thaliana* (Table III). In addition, many others genes (more than 60) are coding for putative polygalacturonases or rhamnogalacturonases which have not been characterised so far. In addition it was found that in tomatoes, several endo-polygalacturonases are present (Pozsar-Hajnal & Polacsek-Racz, 1975). As mentioned above, it is important to stress that the

presence of the genes does not mean that the corresponding enzymes are expressed in the plant but it allows us to speculate on the possible presence of differently acting pectic enzymes. These results may show that many different PME's and endo-polygalacturonases may be present in *Arabidopsis thaliana* showing the importance for the plant to form and/or degrade free GalA blocks over pectic molecules. It seems reasonable to extrapolate this conclusion to citrus plant as well.

Table III: Polygalacturonases in *Arabidopsis thaliana* (Family GH28: Glycoside Hydrolase family 28) as found with CAZy (Carbohydrate Active enzymes, http: //afmb.cnrs-mrs.fr/CAZY/GH_28.html; june 2005) and TIGR (The Institute for Genomic Research, http: //www.tigr.org/tigr-scripts/eik_manatee; june 2005).

Enzyme	Organism	SwissProt	Literature
Polygalacturonase	*A. thaliana*	O65401 Q39094 Q8W4P2 Q9LVJ4	CAZy
Polygalacturonase	*A. thaliana*	O23147	CAZy
Polygalacturonase 3	*A. thaliana*		TIGR
Endo-polygalacturonase	*A. thaliana*		TIGR
Endo-polygalacturonase	*A. thaliana*		TIGR
60 other putative polygalacturonases or rhamnogalaturonases are present in the *A. thaliana* genome (CAZy)			

These two different classes of pectin modifying enzymes (endo-PG and PME) are expressed in different conditions e.g. elongation of the plant cells or formation of thicker cell wall as described in literature (Tucker & Seymour, 2002). Probably, a mixture of different pectins is present showing variation in the amount of non-methyl esterified blocks over the pectin backbone. This mixture may depend on the growing conditions of the fruits and the stage of harvest. It should also be realised that these differences may occur on cell wall level as well as on tissue level resulting in complex mixtures of molecules once pectins are extracted. These speculations are supported in literature since Tucker and Seymour (2002) indicate that pectinases have several isoforms which may reflect differences in substrate activities and they

also indicate that the distribution of specific pectinases such as PG and PME varies both between species and within plant tissues (Tucker & Seymour, 2002).

Another way to verify the presence of free GalA blocks in pectins in plants is to use antibodies specific for pectins. Several antibodies have been recently developed. JIM 5 and JIM 7 are thought to bind to low methyl esterified pectins and high methylesterified pectins respectively (Knox, Linstead, King, Cooper & Roberts, 1990; Willats et al., 2000) but they were shown to have quite some cross-reactivity. The antibody 2F4 recognises dimers of calcium-homogalacturonan complexes (Liners, Letesson, Didembourg & van Cutsem, 1989; Liners, Thibault & van Cutsem, 1992). Two antibodies are specific indicators for the presence of non-methyl-esterified GalA blocks: the PAM 1 antibody recognises sequences of approximately 30 de-esterified GalA residues (Willats, Gilmartin, Mikkelsen & Knox, 1999; Willats et al., 2000), while the antibody LM 7 is claimed to be specific for a random pattern of free GalA (Willats, Orfila et al., 2001). The precise epitopes to be recognised by these antibodies and the localisation of these structures in plant cells has been reviewed recently (Tucker & Seymour, 2002). With this immuno-labelling technique, homogalacturonans are generally found to be distributed throughout the primary cell walls and in the middle lamella but the extend and distribution of methyl-esters was found to vary as reviewed by Tucker & Seymour (2002). Willats et al. showed for sections of pea stem (Willats, Orfila et al., 2001) as well as for *Arabidopsis thaliana roots* and seeds (Willats, MacCartney & Knox, 2001) that pectins present in different cell types show different pectin structures with respect to methyl-ester level and distribution.

The presence of several PME and endo-PG genes in the genome of *Arabidopsis thalina* and the presence of pectins with a different level and distribution of methyl-esters as indicated by immuno-labeling at different positions in the cell wall emphasises the importance of pectin structure for the plants. This also indicates the possibility for the plant to express these different enzymes to modify pectins with respect to their molecular weight, charge, gelling ability with calcium and enzymatic degradability.

2.2. Pectins and their chemical extraction

In our study, commercial pectins are extracted from lemon peel in acid conditions which also affect the distribution of the methyl-esters. Acid and alkaline extraction of pectins are indeed known to de-esterify pectins in a random way (Daas, Meyer-Hansen, Schols, De Ruiter &

Voragen, 1999). It is demonstrated in literature that the nature of acid, the pH, the temperature and the time of extraction of sugar beet pectins modify the degree of methyl-esterification, degree of actelylation and molecular weight of the extracted pectins (Levigne, Ralet & Thibault, 2002). Both enzymatic modifications occurring before the extraction of pectins (in situ) and chemical modifications during the extraction and down stream processing of the pectin can influence the presence and the distribution of the free GalA. Therefore, the origin of the different distribution of the substituents over the pectin is still complex to understand.

2.3. Pectins in acid dairy drinks

The differences between the calcium sensitive pectin A and the non calcium sensitive pectin B were very pronounced in their stabilisation properties of Acid Dairy Drinks (ADD) products. ADD usually consists of a neutral base (milk) with an acidic medium (e.g. fruits). ADD products are stabilised by the addition of sugar and HM pectins to prevent sedimentation of the casein micelles. Laurent et al (Laurent & Boulenguer, 2003) analyzed the sediment formation of the products stabilized with pectins A and B (also used in our study) to determine the effect of the distribution of methyl-esters on the mechanism of stabilization. An important finding was the fact that only the calcium sensitive fraction present in the crude pectin was involved in the stabilization of ADD (Glahn & Rolin, 1996). This emphasises the importance of the characterisation of individual pectic populations present in commercial pectin preparations since some of these populations were found to be more important for the desired physical property in the given application. It was suggested that the higher stabilization properties of the calcium sensitive pectin (especially at low milk concentrations) was due to the larger GalA blocks present at the non-reducing end of the calcium sensitive pectin which bound to the protein (in acid dairy drinks) by a single point attach mechanism. A relatively thick layer of the pectins around the proteins would favour a better stabilisation of the drink compared to multiple attaches of the non calcium sensitive pectin to the protein (Laurent & Boulenguer, 2003). However, from our results, it was found that the binding to the proteins is probably due to the blocks present inside the molecule or at the reducing end but not from the free Gala blocks at the non-reducing end. Furthermore, as shown in chapter 3, the digestion of individual populations with endo-PG results in a rather distinct shift in the Mw as measured by HPSEC which would not have been the case when PG degradable blocks were only located at the non-reducing end.

2.4. HM pectins in gel formation

The gel formation of pectins A and B (0.75%) has been evaluated by the group of Hermansson at pH 3 and 3,5 in presence of sucrose (60%) (Lofgren, Guillotin, Evenbratt, Schols & Hermansson, 2005). It has been noticed that the gelling time at pH 3 is dependent on the distribution of the methyl-esters over the molecule: the more free GalA blocks (and thus methyl-esterified GalA blocks), the faster the gelation time. The non-calcium sensitive pectin B gave a weaker gel compared to the calcium sensitive pectin A. It is suggested that the methyl-ester blocks in commercial pectins may interact stronger through hydrophobic interactions when compared to methyl-esterified residues are distributed at random. This mechanism of blockwise HM pectins would form faster and stronger gels. When the gels formed were examined by microscopy, no differences in the gel structure were observed. Both pectins A and B had a coarse network structure as described previously (Lofgren et al., 2005). Calcium was added for gel formation at pH 3 and the gels of both pectins A and B were different from the ones formed at pH 3 without calcium. The difference in gel strength between the two pectins A and B, as observed for the gels without calcium at pH 3 is less pronounced in the presence of calcium. However, in the presence of calcium, pectin A is forming a gel within few minutes whereas pectin B needs few hours to form a gel. The gel is slightly stronger after 10 hours for pectin B. The structure of the gel as observed by microscopy is more heterogeneous in pectin A and may be a result of the faster gel formation of pectin A whereas the molecules of pectin B have more time to arrange themselves in a more homogeneous way due to the longer gelling time. It is suggested that the addition of calcium to pectins having more blocks of free GalA residues leads to a too fast gel formation since both hydrogen binding and calcium interaction may occur. In the case of the randomly methyl-esterified pectin B, the calcium can bind only weakly since only a few free GalA blocks are present but this small interaction may bring the pectin molecules together enabling interaction through hydrogen binding of the random methyl-esters. At a pH of 3.5 and in the presence of calcium, a completely different physical behaviour for both pectins was observed. Pectin A had a very slow gel formation while almost no gel formation was observed for pectin B. At this pH, more free GalA groups are present in the ionised form creating more electrostatic repulsion between the polymers. The pectin A was able to form a gel very slowly as a result of the calcium interaction with the free GalA blocks while these blocks were present at too low levels in pectin B for such interaction. The mechanism of gelling behavior

through hydrogen bonding is overruled. The use of purified and characterised populations for rheology experiments may result in a better understanding of the gelling mechanisms since the pectic populations present in the crude pectins might interact differently in the gelling mechanism.

3. Amidated pectins

The distribution of amide groups and methyl-esters in amidated pectins remains unclear. The method used to fractionate and characterise HM pectins was used to analyse amidated pectins as well. Amidated pectins from our study were found to be rather heterogeneous. The populations of pectins D and G were found to be heterogeneous and three parameters were found to vary significantly depending on the individual population present in the two commercial LMA pectins: the degree of substitution, the distribution of the substituents (DB) and the ratio of amide groups versus methyl-esters. However no straight correlation was found with the physical properties of the commercial pectins.

Until now the rheological experiments using amidated pectins were performed with crude pectins, not using purified samples. The gelation mechanism remains unclear. The different ratio of amide groups versus methyl-esters may be important for the gelling properties of the amidated pectins since it has been suggested that amide groups play an important role in gelation by promoting hydrogen bonding (Alonso-Mougan, Meijide, Jover, Rodriguez-Nunez & Vazquez-Tato, 2002; Gross, 1979). The gellation of amidated pectins in the presence of calcium and at different temperature has been compared to LM pectins (Lootens et al., 2003). Amidated pectins were found to have stronger gels at pH below 3 compared to the LM pectins. However, the degree of substitution of the LMA pectins and LM pectins used were different, which makes it difficult to interpret the different gelling properties since the pectins present differences in their total charges as well as differences in the nature of the substituents. Our findings and strategies to evaluate pectin structures present in LMA preparations may be used to control the amidation process in a better way and even to come to better functional properties of the end product.

4. Are pectins linked with proteins and polyphenols?

Commercial pectins contain around 1.5 to 3% of proteins (Kravtchenko, Voragen & Pilnik, 1992). The amino acid composition of the proteins in the lemon pectins studied was found to be similar (Kravtchenko et al., 1992) but the proteins were not studied in detail so far. When pectins A and B were fractionated in our study on an anion exchanger into their composite populations, the UV absorbance at 280 nm was recorded. Molecules with aromatic rings e.g. aromatic amino acids in proteins (tyrosine and tryptophane) and polyphenols absorb at this wavelength. Some pectic fractions were found to absorb at 280 nm and this may indicate the presence of proteins in these samples (results not shown). The covalent binding of small amounts of protein to pectin has been suggested earlier by Akhtar et al (Akhtar, Dickinson, Mazoyer & Langendorff, 2002) as a result of their observation that only part of the pectin molecules could stabilise emulsions although no structural information on the protein was presented. Recently, it was suggested that small amounts of arabinogalactan proteins may be covalently attached to pectins isolated from carrot seeds and roots as was demonstrated by precipitation of pectin-AGP complexes with the AGP-specific Yariv-reagent (Immerzeel, 2005). However, the populations obtained in our study did not react with the Yariv reagent suggesting that arabinogalactan proteins were not present in our pectin preparations. Furthermore, it is known from literature that proteins are associated to pectins in the cell wall since proteins like wall-associated kinases can be released after endo-PG digestion of the cell wall (Mort, 2002). It is suggested in literature that the carboxyl groups of GalA residues in pectins may interact with the amino groups of proteins through electrostatic interactions (Mort, 2002) and also hydrogen binding is suggested to be important in protein-pectin complexes in model systems (Girard, Turgeon & Gauthier, 2002).

Another group of compounds which may absorb at 280 nm are phenolic compounds in general. Again, their amount in commercial pectins has been reported to be rather low (<1%) (Kravtchenko et al., 1992). It is known that pectins (mainly HM pectins and RGII dimers) can be associated with some polyphenols (e.g. tannins) (Le Bourvellec, 2003; Riou, Vernhet, Doco & Moutounet, 2002). The mechanism is unclear but hydrogen and hydrophobic interactions are suggested.

5. Pectin analysis in the near future

With our analytical approach we were able to reveal differences in pectin preparations of similar chemical structure. The separation of commercial pectins in pectic populations on preparative scale appeared to be rather time consuming: one day for the separation and collection of the pectic fraction. The further purification process of the populations was time consuming as well (3 days for salt removing). Since the analytical WAX HPLC column was shown to fractionate the pectic populations in the same way as the preparative Source-Q column, we were aiming at the characterisation of the populations fractionated on small scale (1.5 mg of pectin injected on the column). Low concentrations of each fraction obtained will then directly be characterised for DM and the distribution of the methyl-esters using CE before and after endo-PG digestion of the samples. However, the electrophoretic mobility of the samples was highly dependant on the level of salts present in the samples and therefore a desalting step was necessary before analysis on CE. As a first attempt, the populations of the commercial pectin B were separated and fractionated using the analytical WAX column (results not shown). The DM of the fractions of pectin B was then analysed by CE after desalting with centrifugal eppendorf devices (10 kDa membranes). Although still problems concerning reproducibility and recovery of methyl-esters occur, we believe that this approach can be used for the rapid characterisation of a whole range of pectin preparations.

6. Medical applications of homogalacturonan

The determination of the distribution of methyl-esters in pectic molecules is important, not only for food systems as described in our studies but also in medical applications. Since dietary fibers (including pectins) are not hydrolysed by enzymes in the small intestine, they can bind to drugs and influence their absorption and thus their bioavailability (Dongowski, Neubert, Haase & Schnorrenberger, 1996). It has been shown that the DM or the distribution of free and methyl-esterified GalA residues of pectin can influence the transport or permeation of drugs (Dongowski et al., 1996). The interaction of pectins and propranolol (β-blocker) has been studied. Propranolol is a drug used for the treatment of high blood pressure, prophylaxis, migraine or anti-anxyolitic and its transport through an artificial membrane is studied in the presence of pectins. The action of HM pectins with a blockwise or random distribution of the non-esterified GalA was compared. The transport of the propranolol was delayed when the DM of the pectins decreased and a longer delay has been observed for the

pectin with blocks of non-methyl-esterified blocks compared to the pectins with a random distribution of the charges (Dongowski et al., 1996). Food components containing LM pectins and blockwise HM pectins may decrease the bioavailability of propranolol indicating the importance of the distribution of the substituents over pectic backbone in drug interaction.

In general, it can be stated that our study gave us quite some new insights in the complexity of commercial pectin preparations and new tools are presented to characterise individual populations present in the crude mixture. Right now, it seems that the next step should be the large-scale fractionation of commercial pectins into their populations allowing functional characterisation of these populations with respect to their gelling, emulsifying and thickening properties and to link these findings with the chemical fine structure to be established as well.

References

Akhtar M., Dickinson E., Mazoyer J., Langendorff V. (2002). Emulsion stabilizing properties of depolymerized pectin. *Food Hydrocolloids*, *16*, 249-256.

Alonso-Mougan M., Meijide F., Jover A., Rodriguez-Nunez E., Vazquez-Tato J. (2002). Rheological behaviour of an amide pectin. *Journal of Food Engineering*, *55*, 123-129.

Benen J. A. E., Vincken J.-P., Alebeek G.-J. V., (2002). Microbial pectinases. In; *Pectins and their Manipulation, S*eymour G. A., Tucker G. B. ,Blackwell Publishing Ltd.,174-215.

Braccini I., Grasso R. P., Perez S. (1999). Conformational and configurational features of acidic polysaccharides and their interactions with calcium ions: a molecular modeling investigation. *Carbohydrate Research*, *317*, 119-130.

Chen E. M. W., Mort A. J. (1996). Nature of sites hydrolysable by endo-polygalacturonase in partially-esterified homogalacturonans. *Carbohydrate Polymers*, *29*, 129-136.

Daas P. J. H., Alebeek G. J. W. M. v., Voragen A. G. J., Schols H. A., (1999). Determination of the distribution of non-esterified glacturonic acid in pectin with endo-polygalacturonase. In: *Gums and Stabilisers for the food industry;* Williams P. A., Phillips G. O., Wrexham, The Royal Society of Chemistry, 3-18

Daas P. J. H., Meyer-Hansen K., Schols H. A., De Ruiter G. A., Voragen A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research*, *318*, 135-145.

De Figueiredo R. W., Lajolo F. M., Alves R. E., Filgueiras H. A. C. (2002). Physical-chemical changes in early dwarf cashew pseudofruits during development and maturation. *Food Chemistry*, *77*, 343-347.

Dongowski G., Neubert R., Haase H., Schnorrenberger B. (1996). Interactions between food components and drugs. Part 4: Influence of pectins and bile salts on propranolol absorption. *International Journal of Pharmaceutics*, *144*, 233-239.

Fischer M., (1993). Changes in the pectic substances during the ripening of apples. Ph.D Thesis, Zurich, Switzerland.

Girard M., Turgeon S. L., Gauthier S. F. (2002). Interbiopolymer complexing between beta-lactoglobulin and low- and high-methylated pectin measured by potentiometric titration and ultrafiltration. *Food Hydrocolloids*, *16*, (6), 585-591.

Glahn P. E., Rolin C., (1996). Properties and food uses of pectin fractions. In: *Gums and stabilisers for the food industry;* Phillips G. O., Wedlock D. J., Williams P. A., Oxford: Pergamon press, 393-402.

Gross M. O., (1979). Chemical sensory and rheological characterization of low-methoxyl pectin gels.

Guillotin S. E., Bakx E. J., Boulenguer P., Mazoyer J., Schols H. A., Voragen A. G. J. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers*, *60*, 391-398.

Immerzeel P., (2005). Characterization of carrot arabinogalactan proteins. Ph.D Thesis, Wageningen university, The Netherlands.

Ishii S., Kiho K., Sugiyama S., Sugimoto H. (1979). Low-Methoxyl Pectin Prepared by Pectinesterase from *Aspergillus japonicus*. *Journal of Food Science*, *44*, 611-614.

Knox J. P., Linstead P. J., King J., Cooper C., Roberts K. (1990). Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta*, *181*, 512-521.

Kohn R. (1975). Ion binding on polyuronates. Alginate and pectin. *Pure and Applied Chemistry*, *42*, 371-397.

Kohn R., Furda I., Kopec Z. (1968). Distribution of free carboxyl groups in the pectin molecule after treatment with pectin esterase. *Collection of Czechoslovak Chemical Communications*, *33*, 264-269.

Korner R., Limberg G., Christensen T. M. I. E., Mikkelsen D. J., Roepstorff P. (1999). Sequencing of parially methyl-esterified oligogalacturonates by tandem mass spectrometry and its use to determine pectinase specificities. *Analytical Chemistry*, *71*, 1421-1427.

Kravtchenko T. P., Voragen A. G. J., Pilnik W. (1992). Analytical comparison of three industrial pectin preparations. *Carbohydrate Polymers*, *18*, 17-25.

Laurent M. A., Boulenguer P. (2003). Stabilization mechanism of acid dairy drinks (ADD) induced by pectin. *Food Hydrocolloids*, *17*, 445-454.

Le Bourvellec C., (2003). Association entre les procyanidols et les polymers parietaux de pommes: quantification et concequences. Ph.D Thesis, UFR Sciences de la vie et de l'environnement, Rennes, France.

Levigne S., Ralet M. C., Thibault J.-F. (2002). Characterisation of pectins extracted from fresh sugar beet roots under different conditions using an experimental design. *Carbohydrate Polymers*, *49*, 145-153.

Limberg G., Korner R., Buchholt H. C., Christensen T. M. I. E., Roepstorff P., Mikkelsen D. J. (2000). Quantification of the amount of Galacturonic acid residues in blocksequences in pectin homogalacturonan by enzymatic fingerprinting with exo- and endo-polygalacturonase II from *Aspergillus niger*. *Carbohydrate Research*, *327*, 321-332.

Liners F., Letesson J. J., Didembourg C., van Cutsem P. (1989). Monoclonal Antibodies against pectin. *Plant Physiology*, *91*, 1419-1424.

Liners F., Thibault J.-F., van Cutsem P. (1992). Influence of the degree of polymerisation of oligogalacturonates and of esterification pattern of pectin on their recognition by monoclonal antibodies. *Plant Physiology*, *99*, 1099-1104.

Lofgren C., Guillotin S., Evenbratt H., Schols H., Hermansson A.-M. (2005). Effects of calcium, pH and blockiness on kinetic rheological behavior and microstructure. *Biomacromolecules*, *6*, 646-652.

Lootens D., Capel F., Durand D., Nicolai T., Boulenguer P., Langendorff V. (2003). Influence of pH, Ca concentration, temperature and amidation on the gelation of low methoxyl pectin. *Food Hydrocolloids*, *17*, 237-244.

Mort A. J., (2002). Interactions between pectins and other polymers. In: *Pectins and their Modification;* Knox J. P. , Blackwell Publishing Ltd., 30-47.

Nairn C. J., Lewandowski D. J., Burns J. K. (1998). Genetics and expression of two pectinesterase genes in Valencia orange. *Physiologia Plantarum*, *102*, 226-235.

Powell D. A., Morris E. R., Gidley M. J., Rees D. A. (1982). Conformations and interactions of pectins II. Influence of residue sequence on chain association in calcium pectate gels. *Journal of molecal biology*, (155), 517-531.

Pozsar-Hajnal K., Polacsek-Racz M. (1975). Determination of pectinmethylesterases, polygalacturonase and pectics substances in some fruits and vegetables. Part I. Study into the pectolytic enzyme content of tomatoes. *Acta Alimentaria*, *4*, 271-289.

Redgwell R., MacRae E., Hallett I., Fischer M., Perry J., Harker R. (1997). In vivo and in vitro swelling of cell walls during fruit ripening. *Planta*, *203*, 162-173.

Riou V., Vernhet A., Doco T., Moutounet M. (2002). Aggregation of grape seed tannins in model wine-effect of wine polysaccharides. *Food Hydrocolloids*, *16*, 17-23.

Tucker G. A., Seymour G. B., (2002). Modification and degradation of pectins. In: *Pectins and their Modification* ; Seymour G.B. and Knox J.P., Blackwell Publishing Ltd., 150-168.

Willats W. G. T., Gilmartin P. M., Mikkelsen J. D., Knox P. J. (1999). Cell wall antibodies without immunization: generation and use of de-esterified homogalacturonan block-specific antibodies from a native phage display library. *The Plant Journal*, *18*, (1), 57-65.

Willats W. G. T., Limberg G., Buchholt H. C., Alebeek G. J., Benen J., Christensen T. M. I. E., Visser J., Voragen A., Mikkelsen J. D., Knox J. P. (2000). Analysis of pectic epitopes recognised by hybridoma and phage display monoclonal anitbodies using defined oligosaccharides, polysaccharides, and enzymatic degradation. *Carbohydrate Research*, *327*, 309-320.

Willats W. G. T., MacCartney L., Knox J. P. (2001). In-situ analysis of pectic polysaccharides in seed mucilage and at the root surface of *Arabidopsis thaliana*. *Planta*, *213*, 37-44.

Willats W. G. T., Orfila C., Limberg G., Buchholt H. C., van Alebeek G. W. M., Voragen A. G. J., Marcus S. E., Christensen T. M. I. E., Mikkelsen D. J., Murray B. S., Knox J. P. (2001). Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. *The journal of biological chemistry*, *276*, (22 (june 1)), 19404-19413.

Zhan D., Jansson P., Mort A. J. (1998). Scarcity or complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin. *Carbohydrate Research*, *308*, 373-380.

Summary

The aim of this thesis was to extend the analytical toolbox to analyse the chemical structure of pectins in more detail with the hope to be able to explain or even to predict the gelling behavior of these biopolymers more accurately. As summarized in chapter 1, the "standard" analysis of pectins by the manufacturer do not always make distinction between pectins having different gelling, thickening or stabilizing properties. These physical differences can be due to large variations in intramolecular and intermolecular distributions of the methyl-esters over the pectic backbone. Therefore these parameters were analyzed in our study. Commercial pectins were firstly analyzed on an analytical weak anion exchange (WAX) column (chapter 2). The separation was shown to be dependent on the level and distribution of the methyl-esters as observed by comparison of pectins de-esterified in a blockwise manner (with plant PME) and in a random manner (fungal PME). In addition, this column was found to be able to discriminate between two commercial HM pectins known to have similar chemical characteristics by conventional analysis but exhibiting different gelling behavior, in a simple and rapid way. Elution profiles obtained, indicated the presence of several populations within the mother pectins.

Since a detailed characterization of such individual populations required higher amounts of sample, commercial HM pectins needed to be fractionated on a preparative scale. A Source-Q anion exchange column gave an identical fractionation as the analytical WAX column (chapter 3). The "routine" chemical characteristics such as the molecular weight (Mw) distribution and the galacturonic acid (GalA) content were found to be similar for most populations. Information about the distribution of the methyl-esters was obtained by determining the DB_{abs} which is the amount of mono-, di- and triGalA released after endo-polygalacturonase digestion of the pectins, divided by the amount of GalA in the sample (free GalA and substituted GalA). The degree of methyl-esterification (DM) and the distribution of the methyl-esters (DB_{abs}) were different for the various pectic populations. It was also shown that most of the PG degradable blocks were located inside the galacturonan backbone or at the reducing end since digestion of the pectins with an exo-polygalacturonase released only small amounts of free GalA from the non-reducing end. These free GalA blocks at the non-reducing end were found to fluctuate from one pectic population to another although the length of these

small blocks may still be too short to have a large influence on the physical behavior of the pectins.

The second part of this research focussed on commercial amidated pectins obtained by chemical amidation of the HM pectins. The distribution of the methyl-esters and amide groups over the pectin backbone reported in literature is controversial since it is mentioned to be both blockwise and random. This study focussed on the quantification of amide groups and on revealing the precise distribution pattern of the amide groups in amidated pectins.

To be able to determine the degree of substitution (DS), DM and degree of amidation (DAm) of a small amount of samples, a capillary electrophoresis (CE) method was adapted and validated (chapter 4). The CE method separates pectins as function of the total charge: similar eletrophoretic mobilities were observed for pectins substituted to the same degree with either amide groups or methyl-esters. It was concluded that the total charge and not the distribution of the charges determines the electrophoretic mobilities confirming earlier literature. In contrast to CE, the distribution of the charges over the pectic molecules has an effect on the elution from the anion exchanger. Results obtained using CE fitted nicely with results obtained by FTIR (chapter 4). The CE was also used to determine the degree of blockiness (DB) of pectins which was determined so far using HPAEC at pH 5. For this purpose, methyl-esterified pectins were digested with an endo-polygalacturonase and the mono-, di- and tri GalA released were analysed on CE. The DB values obtained using CE were found to be similar to those obtained using the HPAEC method.

The HPAEC method used to determine the DB was further adapted to determine the distribution of the amide groups over the pectic backbone (chapter 5). Amidated pectins were digested with an endo-polygalacturonase and oligomers released were identified using HPAEC and MALDI-TOF MS. The elution from the PA1 anion exchanger was shown to be related to the charge of the oligomers as well as to the nature of the substituents. Considering oligomers of the same size and charge, methyl-esterified oligomers were found to elute before amidated oligomers. Furthermore, the distribution of both amide groups and methyl-esters was found to be rather random in the amidated pectins studied although small differences were found between two commercial pectins.

In chapter 6, the fractionation and characterisation of these two amidated samples having similar chemical characteristics but different calcium sensitivity was described. Different populations were obtained by preparative anion exchange chromatography. Most of the pectic populations had a similar degree of substitution but differed in the ratio of amide groups

versus methyl-esters (Am/Me). This different ratio Am/Me in pectic populations indicated a heterogeneous amidation process of the HM pectins.

In chapter 7, the results of this thesis are discussed and efforts are made to correlate our findings to the physical behavior of these same pectins as published in literature. The various possibilities for the origin of the enormous variation in the chemical fine structure of pectins is discussed with focus on the enzymatic modification of pectins in the plant tissue itself and in the corresponding peel before and during the extraction process.

Samenvatting

Het doel van dit proefschrift was om het analytische gereedschap voor de analyse van de chemische structuur van pectines uit te breiden, zodat het geleergedrag van deze biopolymeren beter kan worden verklaard en zo mogelijk voorspeld. Zoals is samengevat in hoofdstuk 1, maakt de "standaardanalyse" van pectines door de fabrikant niet altijd onderscheid in pectines die verschillende gelerende, verdikkende of stabiliserende eigenschappen bezitten. Deze fysische verschillen kunnen te wijten zijn aan grote variaties in de intra- en intermoleculaire verdeling van methylesters over de hoofdketen en daarom zijn deze parameters in dit onderzoek geanalyseerd. Commerciële pectines zijn eerst geanalyseerd op een zwakke anionwisselingskolom (WAX-10). Door pectines, waarvan methylesters bloksgewijs zijn verwijderd (met plant-PME), te vergelijken met pectines, waarvan methylesters willekeurig zijn verwijderd (met schimmel-PME), is gebleken dat de scheiding afhankelijk is van het gehalte aan en de verdeling van methylesters. Bovendien bleek deze kolom op eenvoudige en snelle wijze in staat onderscheid te maken tussen twee commerciële HM-pectines met vergelijkbare chemische eigenschappen, maar met verschillend geleergedrag. De elutieprofielen duidden op de aanwezigheid van verschillende populaties in beide pectines.

Omdat voor een gedetailleerde karakterisering van zulke individuele populaties grote hoeveelheden materiaal nodig waren, zijn de commerciële HM-pectines op preparatieve schaal gefractioneerd. Een Source-Q anionwisselingskolom vertoonde dezelfde fractionering als de analytische WAX-10 kolom (hoofdstuk 3). Chemische eigenschappen zoals de verdeling van het molecuulgewicht (Mw) en het gehalte aan galacturonzuur (GalA) bleken overeenkomstig voor de meeste populaties. Informatie over de verdeling van methylesters is verkregen aan de hand van DB_{abs}: de hoeveelheid mono-, di- en tri-GalA vrijgemaakt door pectineafbraak met endo-polygalacturonase gedeeld door de totale hoeveelheid GalA in het monster (zowel vrij als gesubstitueerd GalA). De veresteringsgraad (DM) en de verdeling van de methylesters (DB_{abs}) waren verschillend voor de pectinepopulaties. Er is bovendien aangetoond dat de meeste PG-afbreekbare blokken zich in de galacturonzuurketen of aan het reducerende einde bevonden, want bij de afbraak van pectines met een exo-polygalacturonase kwamen slechts kleine hoeveelheden vrij GalA van het niet-reducerende einde vrij. Deze vrije GalA-blokken aan het niet-reducerende einde bleken in omvang te fluctueren tussen de ene en

de andere pectinepopulatie. De lengte van deze kleine blokken is waarschijnlijk te klein om een grote invloed op het fysische gedrag van de pectines uit te oefenen.

Het tweede deel van het onderzoek richtte zich op commerciële, geamideerde pectines, verkregen door chemische amidering van de HM-pectines. Literatuurgegevens over de verdeling van methylesters en amidegroepen over de hoofdketen van pectine zijn tegenstrijdig, omdat deze zowel bloksgewijs als willekeurig wordt genoemd. In dit onderzoek was de nadruk gefocust op het kwantificeren van amidegroepen en het ontrafelen van de precieze verdeling van amidegroepen in geamideerde pectines.

Om de substitutiegraad (DS), de methyleringsgraad (DM) en de amideringsgraad (DAm) te kunnen bepalen van een kleine hoeveelheid monster is een methode voor capillaire electroforese (CE) aangepast en gevalideerd (hoofdstuk 4). De CE-methode scheidt pectines als functie van de totale lading: voor pectines met dezelfde DAm of DM is een overeenkomstige electroforetische mobiliteit waargenomen. Er is geconcludeerd dat de totale lading en niet de ladingsverdeling de electroforetische mobiliteit bepaalt en daarmee werd eerdere literatuur bevestigd. In tegenstelling tot CE heeft de verdeling van lading over pectinemoleculen wel invloed op de elutie van een anionwisselingskolom. Resultaten verkregen met CE voor de DS, DM en DAm kwamen goed overeen met resultaten verkregen met FTIR (hoofdstuk 4). CE is tevens gebruikt om de mate van bloksgewijze verdeling (DB) te bepalen, die tot dusver alleen met HPAEC bij pH 5 bepaald kon vorden. Hiervoor zijn methylveresterde pectines afgebroken met endo-polygalacturonase en zijn de vrijgekomen mono-, di- en tri-GalA geanalyseerd met CE. Ook de DB-waarden verkregen met CE waren overeenkomstig met waarden bepaald met HPAEC.

De HPAEC-methode, die werd gebruikt voor de bepaling van DB werd verder aangepast om de verdeling van amidegroepen over de hoofdketen van pectine te bepalen (hoofstuk 5). Geamideerde pectines zijn afgebroken met een endo-polygalacturonase en vrijgekomen oligomeren zijn geïdentificeerd met HPAEC en MALDI-TOF MS. De elutie van de PA1 anionwisselingskolom bleek te zijn gerelateerd aan zowel de lading van de oligomeren als de soort substituenten. Wat betreft oligomeren met dezelfde grootte en lading, bleken methylveresterde oligomeren vóór geamideerde oligomeren te elueren. Bovendien bleek de verdeling van zowel amidegroepen als methylesters in de bestudeerde geamideerde pectines behoorlijk willekeurig, hoewel kleine verschillen werden gevonden tussen de twee commerciële pectines.

In hoofdstuk 6 is de fractionering en karakterisering van deze twee geamideerde pectines met overeenkomstige chemische eigenschappen maar verschillende calciumgevoeligheid

beschreven. Met preparatieve anionwisselingschromatografie zijn verschillende populaties verkregen. De meeste pectinepopulaties hadden een overeenkomstige substitutiegraad, maar verschilden in de ratio amidegroepen / methylesters (Am/Me). Dit verschil in Am/Me ratio in pectinepopulaties duidt op een heterogeen amideringsproces van HM-pectines.

In hoofdstuk 7 zijn de resultaten van het proefschrift bediscussieerd en zijn pogingen ondernomen de resultaten te correleren aan literatuurgegevens over fysisch gedrag van dezelfde pectines. Verscheidene mogelijkheden voor de oorsprong van de enorme variatie in de chemische fijnstructuur van pectines worden besproken met de nadruk op enzymatische modificaties van pectines in het plantenweefsel zelf en in de bijbehorende by produkten vóór en tijdens het extractieproces.

Résumé

Le but de cette thèse était d'augmenter le nombre d'outils disponibles pour analyser la structure des pectines plus en détail et tenter d'expliquer voire de prédire le comportement gélifiant de ces bio-polymères de façon plus précise. Comme indiqué dans le chapitre 1, l'analyse "standard" des pectines par les industriels ne permet pas toujours de distinguer les pectines présentant différentes propriétés gélifiantes, épaississantes ou stabilisantes. Les différences de ces propriétés physiques peuvent être dues à des variations importantes dans la distribution des groupes méthylés du squelette pectique au niveau intramoléculaire ou intermoléculaire. Ces paramètres ont donc été analysés dans cette étude. Les pectines commerciales ont tout d'abord été analysées sur une colonne analytique faiblement échangeuse d'anions (WAX-10 ; chapitre 2). Il fut démontré que la séparation des pectines sur cette colonne était due à la quantité et à la distribution des groupes méthyles après comparaison des pectines dé-méthylées en blocs (utilisation de la PME des plantes) et de manière aléatoire (utilisation de la PME des champignons). En outre, cette méthode de séparation simple et rapide permet de distinguer deux pectines commerciales hautement méthylées (HM) qui possèdent des caractéristiques chimiques similaires (d'après les méthodes d'analyses conventionnelles) mais des propriétés gélifiantes différentes. Les profils d'élution obtenus ont indiqué la présence de plusieurs populations pectiques au sein des pectines mères. Comme la caractérisation détaillée de ces populations pectiques nécessite de plus grandes quantités d'échantillon, les pectines commerciales HM ont été fragmentées à l'échelle préparative. Une colonne Source-Q échangeuse d'anions a donné une fragmentation similaire à celle obtenue avec la colonne analytique WAX-10 (chapitre 3). Les caractéristiques chimiques "standards" comme la distribution du poids moléculaire (Mw) et le contenu en acide galacturonique (GalA) furent similaires pour la plupart des populations. Des informations sur la distribution des groupes méthylés ont été obtenues en déterminant le DB (degré des substituants en blocs). Le DB correspond à la quantité d'acides mono-, di- et trigalacturoniques, libérés après digestion des pectines (par une endo-polygalacturonase) par rapport à la teneur en acide galacturonique dans les échantillons (GalA libre et substitué). Le degré de méthyl-esterification (DM) et la distribution des méthyl-esters (DB_{abs}) des populations pectiques purifiées furent différents. En utilisant une exo-polygalacturonase libérant peu de GalA non méthly-esterifiés à l'extrémité non réduite des pectines, il fut

démontré que les blocs dégradés par l'endo-PG étaient localisés à l'intérieur ou à l'extrémité réduite du squelette pectique. La taille de ces blocs d'acides galacturoniques libres à l'extrémité réduite fluctua d'une population pectique à l'autre mais cette taille est probablement trop petite pour avoir un effet important sur les propriétés physiques des pectines.

La deuxième partie de cette étude focalise sur les pectines amidées obtenues après amidation chimique des pectines HM. Les résultats concernant la distribution des méthyl-esters et des groupes amidés sur le squelette pectique décrits dans la littérature sont contradictoires : une distribution des substituants en blocs mais également une distribution aléatoire sont suggérés. Notre étude focalise sur la quantification des groupes amidés et sur la distribution de ces groupes dans les pectines amidées.

Afin de déterminer le degré de substitution (DS), le degré de méthylation (DM) et le degré d'amidation (DAm) de faibles quantités de pectines, une méthode a été développée et validée en utilisant une électrophorèse capillaire (CE ; chapitre 4). Cette méthode utilisant la CE sépare les pectines en fonction de leur charge totale : des migrations électrophorètiques similaires ont été trouvées pour des pectines ayant le même degré de substitution. Il fut déduit que la charge totale et non la distribution des charges détermine les déplacements électrophorètiques ce qui confirme les résultats des précédentes publications. Contrairement a ce qui fut observé en CE, la distribution des charges sur les molécules pectiques a un effet sur leur élution lorsqu'un échangeur d'anions est utilisé. Les résultats obtenus avec la CE (DS, DM, DAm) concordèrent avec ceux obtenus par FTIR (chapitre 4). La CE a aussi été utilisée pour déterminer le degré d'acides libres en blocs (DB) dans les pectines. Ce DB était déterminé auparavant par chromatographie échangeuse d'anions (HPAEC) à pH5. Dans ce but, les pectines méthyl-esterifiées ont été dégradées avec une endo-polygalacturonase et les acides mono-, di- and trigalacturoniques libérés ont été analysés par CE. Les valeurs de DB obtenues furent similaires à celles obtenues en utilisant la méthode HPAEC.

La méthode utilisant l'HPAEC pour calculer le DB a été adaptée pour déterminer la distribution des groupes amides sur le squelette pectique (chapitre 5). Les pectines amidées ont été dégradées par une endo-polygalacturonase et les oligomères libérés ont été identifiés en utilisant l'HPAEC et le MALDI-TOF MS. L'ordre d'élution sur la colonne échangeuse d'anion (PA1) fut dicté par la charge des oligomères mais aussi par la nature des substituents. Lorsque les oligomères furent de taille et de charge identiques, les oligomères méthyl-esterifiés ont élué avant les oligomères amidés. De plus, la distribution des groupes amidés

mais aussi des méthyl-esters fut trouvée comme étant relativement aléatoire dans les pectines amidées étudiées et les deux pectines commerciales amidées n'ont montré que de faibles différences.

Dans le chapitre 6, ces deux pectines amidées avec des caractéristiques chimiques similaires mais une sensibilité différente au calcium sont fractionnées et les fractions sont caractérisées. Les différentes populations furent obtenues par chromatographie préparative échangeuse d'anions. La plupart des populations pectiques présentèrent un degré de substitution similaire mais différents rapports entre les groupes amidés et les méthyl-esters (Am/Me). Ces différents rapports indiquent que le procédé d'amidation des pectins HM fut hétérogène.

Les résultats de cette thèse sont discutés dans le chapitre 7 et les possibles corrélations entre les informations de cette thèse et le comportement physique des pectines décrites dans la littérature sont relatés. L'origine de cette énorme variation dans la structure fine des pectines est aussi analysée en insistant sur les modifications enzymatiques des pectines dans les tissus de la plante et dans la peau des fruits avant et pendant l'extraction des pectines.

Acknowledgements

Since most of the persons are reading this page first, I will make a short summary of my thesis in this part as well…. Ok I won't. This part is rather stressful since I do not want to forget anybody. These four years have been really rich scientifically (I enjoyed a lot the trips for conferences in Japan, USA, Italy, Wales!!) and humanly since I meet so many persons from so many different countries.

First I would like to thank my supervisor Henk for his way of guidance that I liked a lot (straightforward and efficient!), his happiness, his support and the garden talks when I was not so optimistic. I want to thank as well my promotor Fons for his availability and his advices.

I am also grateful to the company Degussa Texturants Systems, especially to Karin Born**,** Patrick Boulenguer, Jacques Mazoyer, Georg Schick for their valuable discussions and their support. I am as well thankful to Catherine Renard who informed me about the Ph.D position in the Netherlands and who has always been available for help. I would like to thank as well Anne-Marie Hermansson, Caroline Löfgren and Camilla Lundell (you are a great "crêpes" cooker!) for the nice discussions.

My great colleagues: Chen (the scientific noodle expert and great noodle cooker!), Peter I (I have to show you a festnoze in Bretagne completely different from your techno…), Gerd Jan (I was not homesick since you are often singing the song from Edith Piaf "non rien de rien"!) and Hauke (you are my favorite Calimero!): I will miss you guys! I had a lot of fun and great moments will stay in my mind (especially when Gerd-Jan and Hauke are buying ice cream and chocolate to support me!). I would also like to thank Steph P: ça a commencé par un même prénom (pas pratique à nous différencier au labo) puis par de grands fous rires même dans les moments durs! Avec Fred, Miki et Christophe on a passé beaucoup de week-end mémorables sur Bruxelles (merci les garçons pour ces bons moments!). Je voudrais aussi remercier Franck, Véro et les enfants (vous faites partie de ma famille et votre présence me fût d'un grand réconfort!).
I would also like to thank Sandra (my shopping partner! we keep in touch for sure!), Mirjam (always there when people need you, you are an amazing women!), Wil (thanks for making

my dutch summary and for being always so nice, oh no! except when your stomach is empty…), René V (instead of walking to the south of France, you have to walk next year with Jodie to Bretagne!), Chantal (the baby sitting of Tosca was great even though at 3 years old she was speaking dutch better than me…), my two sweeties Julia and Renate (I will manage to stop in roller skates…), Peter W (I promise, I will not to switch to English when you speak French!), Kerensa (beautiful bride!), Gerrit vK (for giving me a nice surname that also corresponds to him by the way…), Bas (I have to present you my French friends all so nice! Vive la France et la Hollande!), Nathalie (tous les ans on se fait le festival de Gand?), Bram (you suffered with the CE as much as I did…), Laurice (Nantes dans la Bretagne ou pas…éternelle polémique!), Lynn (hey! you are in France now so I will see you often!), Catriona (thanks for your happiness!), Sergio (you had the feeling to be retired after your thesis and I have a similar feeling…), Mark (you can cycle to Bretagne!), Jolanda (thanks for your help and your kindness and see you in France to practice the French of your son!), Miranda (dynamic woman! I enjoyed a lot working with you), Lex (you have to go back to Bretagne when I will be over there!), Ben (thanks for the enzyme info and for your happiness!), Margaret (always helping me when I needed), Edwin (thanks a lot for the help on CE and with MS), Jan (you are always so sympathic!), Jolan, René K for his help when I wanted to throw away the instruments since they were not "always" working. Gerrit B and Jean-Paul for the discussions and their help. I would like also to thank the students who contributed to this thesis: Joke, Janine, Marta, Edna and Nicolas.

I want to thank as well my french friends, always there when I need them: Alex et Régis (la Guyane c'est loin, mais je viendrai vous voir!), Babeth (on a décidé de faire notre thèse à l'étranger et c'était un bon choix!), Audrey (ton séjour en Hollande restera gravé dans pas mal d'esprits…), Sabrina (ca y est je rentre en France et on va pouvoir passer plus de temps ensemble!!), Flo (ma cocotte française en Hollande, j'ai beaucoup apprécié les après-midi shopping à pipeletter), Karine LR (merci pour ton soutien et toutes les belles cartes!), Steph C (j'ai beaucoup apprécié la boîte de bonbons français mais pas autant que la glace au citron de notre jeunesse!), Peg (ma parisienne adorée!), Sev (ma globe trotteuse préferrée!), Lydie (tu es venue en Hollande avec moi, heureusement que tu étais la!), Gilles (casse des verres dans la brioche, oh le gâchis!), Gaëlle (ca y est! c'est fini pour moi aussi!), Céline et David (mes tourtereaux préferrés!), Arno (mon "msneur" préferré!), Flo K (j'ai hâte de vous voir plus

souvent ainsi que bébé Cylia!), Zourata (je viendrai te voir au Burkina!), William and Yaite (on se reverra en France!).

The last but not the least I would like to thank my family. Je voudrais remercier ma grand-mère que j'adore et qui m'a manqué (tu me feras de la langue de boeuf, s'il te plaît?!) ainsi que mon oncle Pierrot, ma tante Christiane et les enfants. Et enfin, le lien le plus fort qui me rappelle en France: je voudrais remercier mes parents et mon petit "grand" frère ("la soeur" revient, attention aux oreilles!) pour leur soutien. Je trouve pas de mot pour vous dire ce que je ressens: j'ai hâte de pouvoir à nouveau être parmi vous!

Stéphanie

Curriculum vitae

Stéphanie Emmanuelle Guillotin was born on 13th of June 1977 in Vannes (France). She obtained her degree of life sciences (DEUG) in 1997 at the university of Southern Brittany and in 1998 she graduated (Licence, BSc) in organisms and population biology in Rennes. In 1999, she passed the 4th year degree (maîtrise) in cell biology and physiology (speciality plant physiology) at the university of Rennes (mention assez bien). Finally, she obtained the MSc degree at Rennes in Plant adaptations and productions (speciality physiology of cultivated plants) in 2000 (mention bien). Thereafter, she came to Wageningen (The Netherlands) and started her Ph.D at the laboratory of Food Chemistry at the Wageningen University, working on the structural features of commercial pectins as described in this thesis. This project was supported by Degussa Texturant systems (Baupte, France). Since april 2005, she is working as a Post-Doc at the laboratory of Food Chemistry at the Wageningen university since april 2005 on a project in collaboration with Sara Lee/ DE.

List of publications

Full papers

Guillotin, S.E., Bakx, E.J., Boulenguer P., Mazoyer J., Schols H.A., Voragen A.G.J., (2004). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different gelling properties, *Carbohydrate Polymers*, 60, 391-398.

Guillotin S.E. Schols H.A. van Kampen J., Boulenguer P., Mazoyer J., Voragen A.G.J. (2003). Analysis of partially amidated and methyl-esterified galacturonic acid oligomers by high performance anion exchange chromatography and matrix-laser desorption-ionisation time of flight mass spectrometry, In Williams P. A., Phillips G. O. *Gums and Stabilisers for the food industry* 12, Wrexham: The Royal Society of Chemistry, 303-310.

Guillotin S.E., Van Loey A., Boulenguer P., Schols H.A., Voragen, A.G.J. Rapid HPLC method to screen pectins for heterogeneity in methyl-esterification. *To be submitted in Food Hydrocolloids*.

Guillotin S.E., Bakx, E.J., Boulenguer P., Schols H.A., Voragen, A.G.J. Determination of the degree of substitution, degree of amidation and degree of blockiness of commercial pectins by using capillary electrophoresis. *To be submitted in Food Hydrocolloids*.

Guillotin S.E., Van Kampen J., Boulenguer P., Schols H.A., Voragen, A.G.J. Degree of blockiness of amide groups as indicator for differences between amidated pectins. *To be submitted in Biopolymers*.

Guillotin S.E., Mey N. Ananta E., Boulenguer P., Schols H.A., Voragen, A.G.J. Chromatographic and enzymatic strategies to reveal differences between amidated pectins on molecular level. *To be submitted in Biomacromolecules*.

Caroline Löfgren, Stéphanie Guillotin, Hanne Evenbratt, Henk Schols and Anne-Marie Hermansson (2005). Effect of calcium, pH and blockiness on kinetic rheological behavior and microstructure of HM pectin gels. *Biomacromolecules*, 6, 646-652.

Abstracts

Guillotin S.E., Bakx E.J. , Boulenguer P., Mazoyer J., Schols H.A. and Voragen A.G.J. (2004). Differences in pectin's structure revealed by the characterization of pectic populations, *X cell wall meeting*, Sorrento, Italy**.**

Catherine M.G.C. Renard, A. Gacel, S. Guillotin, Ch. Massacrier & P. Guillermin (2001). Systematic difference in cell wall structure between tables and cider apples ?. *IX cell wall meeting,* Rotterdam, The Netherlands.

Guillotin S.E., Schols H.A., Ananta E., Bakx E.J., Boulenguer P., Voragen A.G.J. (2004). Chromatographic and enzymatic strategies to reveal differences in saponified amidated pectin's structure. *X cell wall meeting*, Sorrento, Italy**.**

Addendum

The work described in this thesis has been carried out with the financial support from Degussa Texturants Systems (Baupte, France).

Overview of completed training activities

Discipline specific activities

Courses:

VLAG International advanced course: Advanced food analysis (Wageningen, March 2002)

VLAG Summer school glycosciences (Wageningen, March 2002)

Applied Statistics by Dr. W. Hammers (Wageningen, 2002-2003)

Conferences:

Second international symposium: Pectins and pectinases (Rotterdam, The Netherlands, May 2001)

Cell wall meeting in Toulouse (France, September 2001)

Gums and stabilisers for the food industry (Wrexham, Wales, June 2003)

Scientific exchange (Hamburg, Germany, 2004)

Cell wall meeting (Sorrento, Italy, September 2004)

General courses:

PhD student week VLAG (Bilthoven, The Netherlands, 2001)

Food Chemistry PhD trip (USA, November 2002)

Food Chemistry PhD trip (Japan, December 2004)

Additional activities:

Preparation Ph.D proposal

Degussa scientific meetings (2001-2005)

Food Chemistry Seminars (Wageningen, 2001-2005)

Food Chemistry Colloquia (Wageningen, 2001-2005)

Food Chemistry Pectin meetings (Wageningen, 2001-2005)