Thermal and high pressure high temperature processes result in distinctly different pectin non-enzymatic conversions.

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Abstract

Pectin is a common, extremely complex and process sensitive polysaccharide in plant cell walls with many uses as an additive in the food and biomedical industry. Process induced chemical changes in pectin result in various effects on its functionality. An in-depth study is presented of the effects of thermal compared to HP/HT (high hydrostatic pressure combined with high temperature) processing on pectin nano-structure and characteristics. The results obtained emphasized the necessity of taking into account pectin association and conformation in solution when analyzing molecular weight changes. At a pH of 6.3, a decrease in molecular weight was observed for both thermal and HP/HT treated samples but with partially different reasons. While for the thermally treated samples the reduction in molecular weight was mostly due to pectin depolymerization for the HP/HT treated samples a significant effect was observed for conformational changes induced by electrostatic repulsion caused by the complete demethoxylation of the polymer. On the contrary due to conformational changes, an increase in the observed molecular weight was noticed for HP/HT treated samples at a pH of 4.4. The study also clearly shows the necessity of combining an absolute molar mass determination method like multi angle laser light scattering (MALLS) in studies on the effect of processing on pectin.

Keywords

Pectin, processing, molecular weight, high pressure high temperature, SEC-MALLS

1. Introduction

Pectin, a heterogeneous group of polysaccharides, is structurally and functionally the most complex polysaccharide in plant cell walls with various functions during plant growth and development. It also has a diverse range of food and biomedical uses (Mohnen, 2008). Pectin is composed of approximately 70% galacturonic acid (GalA) and is suggested to be a triad component encompassing homogalacturonan (HG), rhamnogalacturonan II (RG-II), and rhamnogalacturonan I (RG-I) domains (Mohnen, 2008). Generally, the pectin backbone is mostly considered to be composed of both HG and the core of RG-I, the latter being branched with neutral sugar side chains (Coenen, Bakx, Verhoef, Schols, & Voragen, 2007), although alternative models have been suggested. HG is a linear chain of galacturonic acid residues which can be methoxy-esterified at C-6 and/or acetylated on O-2 and O-3 (Vincken, 2003). Changes in fruit/vegetable texture during ripening, processing, and storage are mainly related to (bio-) chemical conversions in pectin. This polysaccharide is abundant in plant tissue middle lamella where it plays a critical role in cell-cell adhesion. Pectin is more chemically reactive than other cell wall polymers (Buren, 1979). In our diet, a range of processed products contain pectin ranging from fruit and vegetable juices (Sila et al., 2009), purees and pastes (Van Buggenhout, Sila, Duvetter, Van Loey, & Hendrickx, 2009) where pectin originates from the raw material up to products such as yogurt, jams and many others where pectin is added as a thickener, stabilizer or gelling agent (Corredig, Kerr, & Wicker, 2000; Corredig & Wicker, 2001). Processing of plant-based foods aims to prolong the shelf life while the original sensory and nutritional properties are maintained as high as possible. Additionally the processing is used to increase the product edibility and palatability (Hendrickx, Oey, Lille, & Van Loey, 2008). Thermal treatment of food is the most common means of preservation (Awuah, Ramaswamy, & Economides, 2007). It is known to modify pectin both enzymatically (through modification of enzyme activity, which is not in the scope of this work) and non-enzymatically. The main non-enzymatic conversions are depolymerization and demethoxylation. The direct non-enzymatic effect is known to be pH dependent. While at neutral to alkaline pH pectin has been shown to break down by a β-elimination reaction with a formation of an unsaturated bond absorbing at 235 nm (Albersheim, Neukom, & Deuel, 1960), at acidic conditions (pH<4.5) acid hydrolysis is increasingly important (Fraeye et al., 2007). Both depolymerization reactions were shown to be

enhanced by temperature (Fraeye et al., 2007). A prerequisite for β-elimination is the presence of a methyl ester group at C-6 rendering H-5 sufficiently acidic to be removed by an alkali. Therefore as the chemical demethoxylation, which requires similar conditions to the β-elimination, proceeds, it reduces the rate of β-elimination due to removal of the methyl ester group (Fraeye et al., 2007). High pressure processing (HPP) is suggested as a processing method that has a minimal deleterious effect on food quality attributes such as color, flavor and nutritional value. It also has the advantage of being transmitted uniformly and instantaneously throughout the food (De Roeck et al., 2009). HPP is known to enhance demethoxylation (Verlent et al., 2004) most likely by the Le Chatelier principle that states that any phenomenon that is accompanied by a decrease in volume is enhanced by an increase in pressure. In the case of pectin demethoxylation, the solvation of the charged groups created is accompanied by a reduction in reaction volume resulting from electrostriction (i.e. the compact alignment of water dipoles owing to the coulombic field of the charged groups) (De Roeck et al., 2009). Without increasing temperature, HPP is not capable of inducing β-eliminative cleavage of pectin chains (Kato, Teramoto, & Fuchigami, 1997). Although HPP is currently commercially applied for a range of pasteurized products, to achieve complete inactivation of enzymes, vegetative micro-organisms, as well as spores, high pressure must be combined with a second inactivating factor such as temperature. By utilization of the compression heat upon pressurization a rapid and uniform heating can be achieved (De Roeck et al., 2009). Only limited information is available regarding the effect of high pressure with high temperature (HP/HT) on pectin. In a study that followed the release of methanol and the formation of unsaturated uronides in a MES (2-(N-morpholino) ethanesulfonic acid) buffer (pH 6.5) at 110 °C it was shown that the rate of release of methanol was increased under pressure (in comparison to atmospheric pressure at the same temperature) reaching the maximum in a few minutes while the formation of unsaturated uronides was retarded, and only occurring in the first few minutes of the treatment. The limited β-elimination observed can be explained both by the rapid decrease in the methyl ester groups required for this reaction, and by the fact that β-elimination is accompanied by an increase in reaction volume (De Roeck et al., 2009).

Size exclusion chromatography (SEC) is a common analytical separation method for studying molecular weight distribution profiles of polysaccharides for research and quality control (Corredig & Wicker, 2001). Upon separation of the molecules by their hydrodynamic volume

(Kravtchenko, Voragen, & Pilnik, 1992a), several methods of detection can be used for approximation or calculation of the molecular weight and radii of the molecule. The approximation of molecular weight by a refractive index (RI) detection of the polysaccharide concentration requires a comparison of the obtained elution profiles to the elution profiles of molecular weight standards. Such methodology, while very commonly used in pectin research (Chen et al., 2012; Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011; Munarin, Bozzini, Visai, Tanzi, & Petrini, 2013), is limited by the fact that the structure of the standards might differ significantly from the structure of the studied pectin resulting in an approximated molecular weight. This point was raised when light scattering of apple and lemon pectins showed different molecular weights for identical elution volumes (Kravtchenko, Berth, Voragen, & Pilnik, 1992). If only RI detection was used, based on a single calibration, such differences could not be detected. The usage of a combination of an RI detector with a multi angle laser light scattering (MALLS) detector eliminates the need of column calibration allowing accurate determination of the molecular weight and commonly of the radii of the polysaccharide (Corredig et al., 2000). A previous work has concluded that size exclusion chromatography, but only with the combined use of MALLS and RI detection, is an effective means to quantify molecular weight changes after homogenization (Corredig & Wicker, 2001). In a different work a right shift in the concentration elution profile of tomato serum pectin due to processing was observed, indicative of a decrease in molecular weight when concluding based on RI only. When taking also into account MALLS data no significant changes in the molecular weight were observed, most likely due to variations in conformation of the pectins (Diaz, Anthon, & Barrett, 2009).

When studying the effects of processing on pectin depolymerization one should keep in mind several pectin properties that, although representative to the true behavior of pectin in solution, might complicate the analysis and conclusions. Both industrial (and research grade) pectin and obviously products directly originating from fruits and vegetables include residual (in case of industrial) or significant concentrations of other molecules (covalently or non-covalently bound) such as proteins and polyphenols (Kravtchenko, Penci, Voragen, & Pilnik, 1993). Additionally, inter- and intra-molecular pectin interactions might also play a role in solution behavior. When pectin is dissolved in water, aggregates are usually present even when no or only monovalent salt

is added (Ousalem, Busnel, & Nicolai, 1993). It was suggested previously that pectins in low ionic strength solutions exist as microgel aggregates (Fishman et al., 1992) and that the presence of NaCl dissociates the microgels by disruption of hydrogen bonding, but even at high ionic strength solutions, aggregates were still suggested to exist, possibly due to other non-covalent interaction (Corredig et al., 2000; Fishman, Cooke, Hotchkiss, & Damert, 1993). Additionally, it was suggested that the SEC itself does not influence the microgel structure (Corredig et al., 2000). Most likely due to this reason in addition to the well-known variation of pectin stability as a function of pH, a controversy exists regarding the effect of SEC elution medium on the molecular weight distribution. A previous study revealed that the buffer that is used for size exclusion has a significant effect on the calculated molecular weight, suggesting that in different buffers, while still soluble, pectin has different levels of association (Fishman, Chau, Kolpak, & Brady, 2001). Contrary to this result, in an earlier study, no difference in molecular weight distribution was found for samples eluted by $NaNO_3$ (pH 5.8) compared to identical samples eluted by $NaNO_3$/phosphate (pH 7) buffer (Corredig et al., 2000). Similarly, no difference in elution chromatograms was seen between apple and lime pectins eluted by 0.1M $NaNO_3$ and 0.1M acetate buffer (Brigand, Denis, Grall, & Lecacheux, 1990). No information was given regarding the ionic strength of the eluents which might also affect the result.

It is well known that quality characteristics of plant based foods, specifically textural and rheological properties, strongly depend on the pectin content, composition and structure. (Sila et al., 2009) Due to the fact that the structure of pectin is significantly affected by processing, therefore strongly modifying its functionality, combined with knowledge gaps regarding process induced non-enzymatic structural pectin changes (especially by novel processing methods like HP/HT), the goal of this work is to provide an in depth view on the effect of HP/HT compared to the effect of thermal processing on pectin characteristics. An integrated analytical approach considering SEC-MALLS-RI-UV is used, allowing also to better understand methodological issues related to determination of molecular weight of pectins.

2. Materials and Methods

2.1 Materials

Apple pectin with a reported degree of methoxylation (DM) of 70.7 was purchased from Sigma-Aldrich (product number: 76282, LOT 1424480), dissolved in water overnight and dialyzed for 48 hours (molecular weight cutoff of 12-14 kDa) against demineralized water, prior to freeze drying, to minimize the presence of small co-solutes. This process was performed several times and all the obtained freeze dried pectin was mixed together for all the experiments in this work. Other reagents used were of analytical grade.

2.2 Methods

2.2.1 Sample preparation

Two 0.1M buffer solution of pH 6.3 and 4.4 were prepared using NaOH titrated MES (2-(N-morpholino) ethanesulfonic acid) and acetic acid respectively. For each processing treatment a new batch of starting 0.2% (w/v) pectin solution was prepared by dissolving the pectin powder in the buffer overnight.

2.2.2 pH corrections

Changes in pressure and temperature can alter the pK_a of weak acids and bases which can lead to very significant pH shifts in samples undergoing the studied processes. To account for this phenomenon, just prior to the processing stage, the pH of the solution was corrected such that under the tested pressure and temperature the starting pH of the buffers (i.e., 4.4 and 6.3) were maintained. The exact value of the corrected pH is noted in each process subsection. The pH correction prior to the processing stage was done using 2M NaOH at a room temperature in a beaker with a magnetic stirrer, while in order to bring the pH back to the starting value immediately after the processing stage 2M HCl was used. In all cases the effect of such pH

correction cycle on the concentration of pectin was extremely small (less than 1%) and in any case all samples for the same processes and pH went through the pH correction cycle. The thermal stability of acetic acid buffer is very high, $\Delta pK_a/\Delta T$ of 0.0002 (Holtzhauer, 2006), therefore no pH correction was performed for the samples thermally treated at pH 4.4. For the thermally treated samples at the pH of 6.3 and for the high pressure high temperature samples at both pH values the correction was performed. For the thermally treated samples at the pH of 6.3 the correction was based on $\Delta pKa/\Delta T$ of 0.011 for MES buffer. For the evaluation of the target pH for correction of the samples under HP/HT conditions the calculation was based on the method of Bruins, Matser, Janssen, and Boom (2007). For the suggested calculation necessary values for both buffers (the pH at atmospheric pressure, the reaction volume ΔV_0, pKa_0, $\Delta pKa_0/\Delta T$) were obtained from previous literature (Bruins, Matser, Janssen, & Boom, 2007; Holtzhauer, 2006; Orlien, Olsen, & Skibsted, 2007). The subscript 0 denotes the value at atmospheric pressure. First, the shift in pKa due to the high temperature and high pressure has to be calculated. While the new pKa at high temperature was obtained using $\Delta pKa_0/\Delta T$ from literature, to compute the pKa at high pressure the following formula (Equation 1) was used (El'yanov & Hamann, 1975):

$$\ln\left(\frac{K_a}{K_a^0}\right) = -\frac{P\Delta V^0}{RT(1+bP)} \quad (1)$$

where P denotes pressure (MPa), R the universal gas constant (8.314 cm^3*MPa *mol^{-1} *K^{-1}), T the absolute temperature (K), and b a universal constant ($9.2 \cdot 10^{-4}$ MPa^{-1}).

Irrespective of the parameter for which the pKa is corrected first, some basic assumptions have to be made. In case the pKa is corrected first for temperature, it is assumed that the ΔV_0 does not change with temperature. In the other case, it is assumed that the $\Delta pKa/\Delta T$ does not change with pressure. We have decided to correct first for the temperature. The obtained K_a can then be used with equation 2:

$$K_a = \frac{[H^+][A^-]}{[HA]} \quad (2)$$

As this equation has too many unknowns to be solved, the molarity of the buffer solution and a mass balance on the hydrogen atoms can be used as a boundary condition. The total amount of hydrogen should remain the same under HP/HT as under atmospheric pressure/room temperature. Finally, the $[H^+]$, and thus the pH can be calculated by iteration. The suggested calculations are still limited by not taking into account the buffering capacity of pectin itself due to its many carboxyl groups (De Roeck et al., 2009).

2.2.3 Processing treatments

In addition to the two processes described in 2.2.3.1 and 2.2.3.2 an original unprocessed 0.2% (w/v) solution of apple pectin (at both buffers described in 2.2.1) was characterized. Before analytical procedures the samples were equilibrated for 2 h in a 25 °C water bath.

Because one of the objectives of this work was to test whether after the filtration step necessary for SEC-MALLS the filtered sample is still representative of the original one, a part of the received sample was filtered through 0.45 μm (Chromafil A-45/25, 0.45 μm, Macherey-Nagel Gmbh, Duren, Germany) after the post-processing equilibration for 2 h at 25 °C.

2.2.3.1 Thermal Treatment

A 0.2% (w/v) solution of apple pectin (at both buffers described in 2.2.1) was divided over screw-capped test tubes (5 ml per tube) and was heated to 100 °C in a thermostated oil bath for 0, 10, 20, 40 min. The time 0 solution (T=0) was defined as the solution after an equilibration period of 5 min that allowed the samples to reach the target temperature of 100 °C. The samples were immediately cooled in an ice water bath, followed by combining the solutions from 6 test tubes and further equilibration of 2 h in a 25 °C water bath before analytical procedures. Thermal treatments were carried out twice starting from a single batch of the apple pectin solution to minimize non process variations. The pH of the sample at the starting pH of 6.3 was

corrected just prior to the thermal treatment to the pH of 7.1 (as described in 2.2.2). After the thermal treatment and cooling the pH was corrected back to 6.3 using HCl.

2.2.3.2 High Pressure High Temperature

The HP/HT treatments were carried out in a custom-made laboratory scale high-pressure unit (Resato, The Netherlands), consisting of six individual vessels (6 × 43 mL, internal diameter = 20 mm), each surrounded by a heating coil connected to a thermostat. The pressure medium consisted of 100% propylene glycol. The teflon (polytetrafluoroethylene) sample holders (12 mm inner diameter, 85 mm length, 4 mm wall thickness, Vink, Belgium) were filled with the 0.2% (w/v) solution of apple pectin sample, closed with a movable cap and vacuum sealed with double plastic bags. The equipment allows computer-controlled pressure build-up and data logging of both pressure and temperature, detailed information can be seen in De Roeck et al. (2009) and in Kebede et al. (2013). In short, first the samples were allowed to heat to 60°C, while in this stage the preheating was the result of heat transfer from the pressure medium to the samples. Upon reaching this temperature the pressure was built up very fast; increasing in 5 s from 0.1 to 150 MPa and then to the set pressure of 600 MPa at a rate of 10 MPa/s. This was accompanied by a temperature rise of the sample to the required temperature of 100 °C due to compression heating. After the desired pressure was reached the individual vessels were isolated. After the desired treatment time (10, 20, 40 minutes) the individual vessels were decompressed and the samples were immediately cooled in an ice water bath, followed by mixing of the samples from 3-4 sample holders. The combined samples were allowed to equilibrate for 2 h in a 25 °C water bath before further analytical procedures. The combined solution from 3 or 4 sample holders (at identical pH) was considered as one repetition, and two repetitions were analyzed. The time 0 solution is defined as the solution that was allowed to reach 60 °C (the temperature before pressure built up). All runs started from a single batch of the apple pectin solution (at each pH) to minimize non process variations. The pH of the sample with the original pH of 6.3 was corrected just prior to the treatment to a pH of 6.92 (as described in 2.2.2). After the treatment and cooling, the pH was corrected back to 6.3. Similarly the pH of the sample with the original pH of 4.4 was corrected to 5. After the treatment and cooling the pH was corrected back to 4.4.

256 2.2.4 Pectin characterization

257 2.2.4.1 Determination of the degree of methoxylation of pectin using Fourier Transform Infra-
258 Red (FTIR)

259 The method is based on measuring the ratio between the absorption intensity of the band situated
260 around 1740 cm^{-1} (due to ester carbonyl group (C=O) stretching) to the sum of the peak
261 intensities at 1740 cm^{-1} and the peak located at 1630-1600 cm^{-1} (due to carboxylate group
262 (COO^{-}) (Szymanska-Chargot & Zdunek, 2013) and comparing it to a calibration data set of
263 pectins with known degrees of methoxylation. In this work the calibration data set included 36
264 points with different DMs. The different DMs were obtained by two methods and combined to
265 one calibration curve. The first set was based on mixing commercial citrus pectin (DM ~ 94%,
266 Sigma-Aldrich, Belgium) with polygalacturonic acid (PGA, Sigma-Aldrich, Germany) in
267 different ratios allowing a large DM range of 0 to 94%. The second set contained pectins
268 demethoxylated either chemically (NaOH) or enzymatically (pectin methyl esterase, PME from
269 carrots, *Daucus carota* cultivar Nantes) to create the various DMs (Ngouémazong et al., 2011).
270 The calibration curve was obtained by linear regression of the plot of colorimetrically
271 determined DMs for the demethoxylated pectin samples or theoretically determined DMs for the
272 mixtures of PGA and citrus pectin against the FT-IR results. The colorimetric method was based
273 on calculating the ratio of the molar amounts of methylesters to the molar amounts of GalA.
274 GalA content was determined by hydrolyzing pectin with concentrated sulfuric acid followed by
275 dilution with demineralized water and concentration determination by a spectrometric method
276 (Blumenkrantz & Asboe-Hansen, 1973). The samples for methanol quantification were
277 hydrolyzed under alkaline conditions to release methanol that was enzymatically oxidized to
278 formaldehyde. The methanol content was subsequently quantified calorimetrically based on the
279 formaldehyde formed (Klavons & Bennett, 1986). The obtained ratio (R) between the intensity
280 of the peak at 1740 to the combined intensities of the peak at 1740 with the peak at 1600 was
281 used to predict the DM of the samples based on the obtained calibration line: DM (%) =
282 136.86*R+3.987. The samples (with and without a filtration step) were dialyzed (molecular
283 wright cutoff 3.5 kDa,) against demineralized water for 48 h, lyophilized, and stored over P_2O_5
284 until measurement. The pH of samples originally at 4.4 was corrected to 6.2 - 6.3 prior to

dialysis to ensure total ionization of the carboxylic groups (Manrique & Lajolo, 2002). From the dry material a small sample was firmly compacted to expel entrapped air and ensure smooth surfaces, 100 scans were run per sample placed on the sample holder of the attenuated total reflectance fourier transform infra-red spectrometer (ATR-FTIR, Shimadzu FTIR-8400S, Japan) and the transmittance was recorded at wavenumbers from 4000 cm^{-1} to 400 cm^{-1} at resolution 4 cm^{-1}. The spectra were converted into absorbance mode before base line correction and reading of the absorption at the maxima of both peaks (1740 and 1600cm^{-1}). .

2.2.4.2 UV-Vis spectral absorbance

The absorbance at 235, 280, 600 nm of the processed pectin samples was measured before and after filtration and the equilibration at 25 °C using a spectrophotometer (Ultrospec 2100 pro, GE Healthcare).

2.2.4.3 Determination of molecular weight distribution by SEC-MALLS-UV-RI

The molecular weight distribution of pectin was determined using size exclusion chromatography (SEC) coupled to multiangle laser light scattering (MALLS) (PN3621, Postnova analytics, Germany), refractive index (RI) (Shodex RI-101, Showa Denko K.K., Kawazaki, Japan) and an a diode array detector (G1316A, Agilent technologies, Diegem, Belgium). 100 µL of 0.2% (w/v) pectin solution after various processing treatments was filtered through a 0.45 µm filter and injected, using an autosampler (G1329A, Agilent technologies, Diegem, Belgium), to a series of three Waters columns (Waters, Milford, MA), namely, Ultrahydrogel 250, 1000, and 2000 with exclusion limits of $8*10^4$, $4*10^6$, and $1*10^7$ g/mol, respectively. Two eluents were used based on the starting pH of the sample: 0.1M acetic acid buffer (pH 4.4) with 0.1M NaCl and 0.1M MES buffer (pH 6.3) with 0.1M NaCl. NaCl was used instead of the commonly used less corrosive $NaNO_3$ (Fishman et al., 2001) to enable the collection of the UV absorbance at 235 nm in order to follow the formation of β-elimination products. Eluents were prepared using demineralized water (organic free, 18 MΩ cm resistance), filtered (0.1 µm) and degased by an on-line degasser of the HPLC system (Agilent technologies 1200 Series, Diegem, Belgium). The flow rate was 0.5 ml/min and the columns were kept at 35 °C. Before injection all samples were

allowed to equilibrate overnight after filtration. A dn/dc value of 0.146 mL/g was used for all samples, this value was found not to differ between $NaNO_3$ and LiAc/HAc buffer (Fishman et al., 2001). Similarly no significant difference in the dn/dc values of pectin was found between 50 mM nitrate (pH 5.8) and 50 mM nitrate/10 mM phosphate (pH 7) buffers (Corredig et al., 2000). The molecular weight was calculated using the Debye fitting method (up to 2nd order) of the software provided by the MALLS detector manufacturer (Nova Mals, version 1.0.0.18, Postnova analytics, Germany). No root mean square radii data are reported due to the relatively small size of the pectin used (30-100 kDa as stated by the manufacturer) resulting in a poor molecular size (radii) detection while the molecular weight values are reliable as was shown for polystyrene in toluene (Barth & Yau, 1989). A similar methodological issue has caused an exclusion of root mean square radii from analysis even for industrial pectins with an average molecular weight exceeding 100 kDa (Corredig & Wicker, 2001). The presented elution profiles are averaged chromatograms of two repetitions.

2.2.4.4 Determination of reducing ends

The concentration of reducing end groups formed was quantified in filtered and non-filtered samples. This assay was performed by heating the solutions (10 min, 100 °C) in presence of cyanoacetamide at pH 9.0, and measuring the absorbance at 276 nm (Ultrospec 2100 pro, GE Healthcare) as described by Gross (1982), with some modifications. Because the method requires heating at pH 9.0, β-elimination will occur that is dependent on the initial DM of the pectin and results in an erroneous increase in the reducing ends measurement. Since β-elimination only occurs next to an esterified galacturonic acid residue (Keijbets & Pilnik, 1974) the magnitude of the error will be correlated with the starting DM of a sample. Therefore prior to the assay, to prevent formation of reducing groups during heating of the reagent, the samples were demethoxylated (Krall & McFeeters, 1998) using 2M NaOH for 1 h at room temperature followed by reducing the pH back to the starting point using 2M HCl. Such method is not considered to cause a measurable increase in the reducing ends (Krall & McFeeters, 1998), while preventing further β-elimination during the heating stage. A standard curve was made using galacturonic acid (Kravtchenko et al., 1993).

2.2.4.5 Protein content

Protein content was determined using an automated Dumas protein analysis system (EAS, varioMax N/CN, Elt, Gouda, The Netherlands); 6.25 was used as the nitrogen to protein conversion factor. The samples of 0.2% (w/v) pectin were prepared in water (organic free, 18 MΩ cm resistance) and the pH was corrected to 6.3 and 4.4 using NaOH and HCl. At both pH two samples of 150 ml were prepared with and without the stage of filtration. The four obtained samples were freeze dried prior to analysis. In this case direct pH correction was selected instead of the usage of buffers to minimize the presence of non-pectin molecules in the dry material after lyophilization.

2.2.4.6 Data analysis

Data reported are the mean and standard error values of the samples. Where significance is reported, the data were subjected to one-way analysis of variance (ANOVA) followed by means comparison using Tukey's multiple comparison test (Origin 8, OriginLab, MA, USA) and were considered significant at $p < 0.05$ (Vervoort et al., 2012)

3 Results and Discussion

To obtain information regarding the molecular weight of macromolecules using SEC-MALLS, a filtration step is required to remove large aggregates. This is done to reduce the risk of blocking the columns and detectors but also because it was shown that very large molecular weight components invade various size exclusion fractions resulting in significant effects on the calculated molecular weight due to the dominant effect of the very large compounds on light scattering measurements (Kravtchenko, Berth, et al., 1992). But such filtration or other "purification" methods that were reported to be used (like centrifugation) might cause the filterable material not to be representative of the original system. It was shown that such filtration reduced up to 60% of the recovery of citrus pectins after filtration without added calcium and by 93% with calcium addition that caused the formation of unfilterable particles (Beaulieu, Corredig, Turgeon, Wicker, & Doublier, 2005).

Therefore to gain some insight regarding the effect of filtration and possibly to have a clearer view about how representative the filterable pectin sample is in respect to the original, in all the experiments possible the measurements were done both on the filtered and unfiltered sample. Additionally, differences between the results obtained on the starting samples (without the processing stage) at both pH can help provide additional data about the effect of the solvent on the conformation of pectin/associated pectin molecules.

3.1 The effect of processing on the pectin UV – VIS absorption

In figure 1 the relative absorbance (compared to the absorbance of the unfiltered sample in starting condition of 0 treatment) is shown.

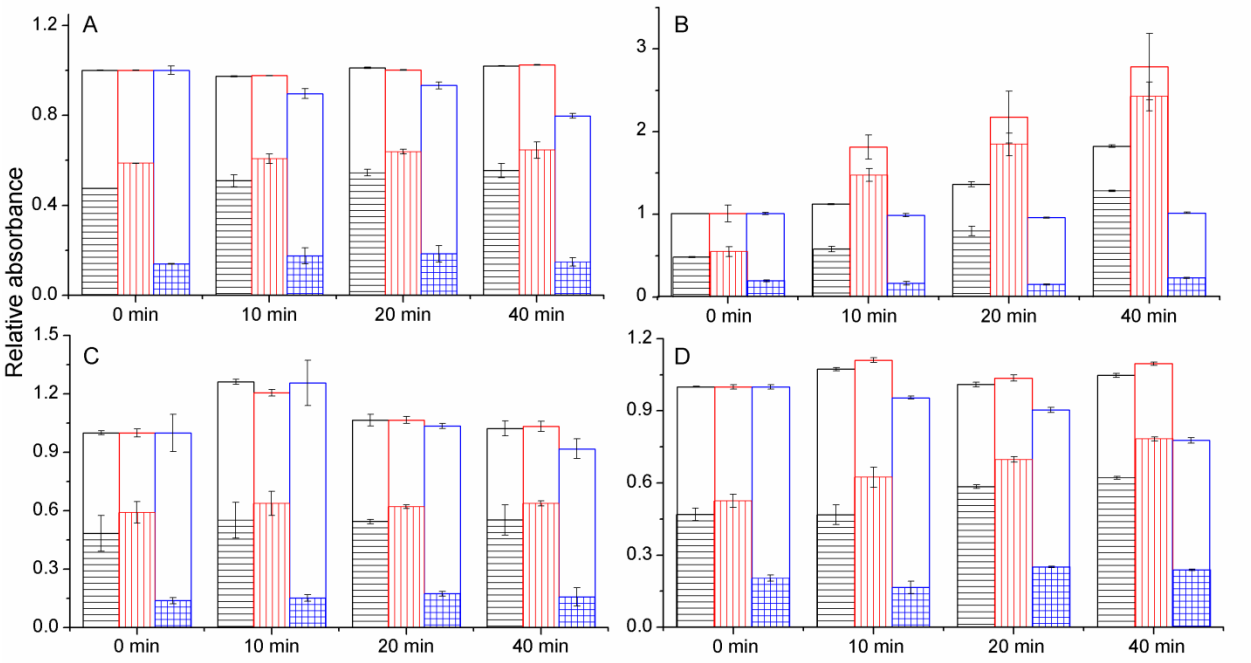

Figure 1: Relative absorbance compared to the absorbance of the unfiltered sample in the starting condition (defined treatment time=0). The empty columns of the same color represent the relative absorbance (compared to starting conditions) of the unfiltered sample. The columns including a pattern inside the empty columns, represent the relative absorbance (compared to the unfiltered sample at the starting condition) of the same sample after filtration. Black, horizontal lines: 280nm ; red, vertical lines: 235nm; blue, chequered: 600nm. A – thermal treatment pH 4.4; B – thermal treatment pH 6.3; C – HP/HT pH 4.4; D – HP/HT pH 6.3.

In all treatments it is clearly seen that the absorbance at 600 nm is the most affected by filtration. The absorbance at 600nm can be related to the solution turbidity (Zimet & Livney, 2009) and therefore it is expected that the filtration that removed the larger particles in the solution also causes the significant reduction of ~80% in the turbidity of the system. The absorbance at 235 nm was used to monitor the formation of β-elimination products while the absorbance at 280 nm, although still affected by the peak at 235 nm can give some information regarding proteins (Whitaker & Granum, 1980). For the absorbance at 280 nm and 235 nm the filtration reduces 60-40% of the absorbance. Since it is very likely that the absorbance at both wavelengths is at least partially related to the presence of protein, the concentration of proteins in the sample before and after filtration was determined using an automated Dumas method at both pH, as can be seen in Table 1.

Table 1: The protein content (%w/w) in pectin with and without a filtration step at pH of 4.4 and 6.3. The results are presented as a Mean ± standard deviation.

Sample	%Protein	
	Unfiltered	Filtered
pH 4.4	1.46±0.34	1.27±0.11
pH 6.3	1.51±0.21	1.20±0.38

Firstly, from the results it is clear that our samples contain limited protein concentrations that are similar to what was obtained for industrial apple pectin (1.6%) in a previous work (Kravtchenko, Voragen, & Pilnik, 1992b). These researchers also stated that further purification by copper precipitation in an attempt to reduce the protein concentration did not result in any reduction in the protein content of the apple pectin. Due to these methodological limitations we also refrained from attempts to further purify the pectin in terms of protein concentration. Additionally, it can be seen that the filtration slightly but not significantly reduces the protein content but most likely it cannot by itself explain the reduction of 40-60% in the absorbance at 280 and 235 nm. It can also be concluded that most likely the aggregates that are removed by filtration (and result in the decrease of 80% in turbidity) are not caused by some protein rich pectin-protein complexes. It is still not fully clear whether the proteins found in purified pectins are covalently linked to the pectin (Oosterveld, Voragen, & Schols, 2002) or not (Ridley, O'Neill, & Mohnen, 2001). One should keep in mind that processing can induce conformational changes that will lead to changes in the UV spectra of proteins (Donovan, 1969). For example the absorbance at 230 nm was suggested to be a valid probe for protein unfolding (Liu, Avramova, & Park, 2009).

In Figure 1A the results for the thermally treated pectin at pH 4.4 are presented. It is well known that around this pH pectin solutions are relatively stable for thermally induced changes (Axelos & Branger, 1993). This fact can also be seen in our results where the absorbance is mostly unaffected by increasing thermal treatment time except of some decrease in the absorbance at 600 nm without filtration possibly due to some improved solubility of a part of the larger particles. Additionally a little increase (of 10%) in the absorbance at 235nm of the filtered sample was noted (although not clearly visible in the figure), with no corresponding increase in

the unfiltered sample. The reason for this increase is not fully clear and it might be related to some limited β-elimination at this acidic pH as was previously suggested for citrus pectin at the pH of 4.5 (Diaz, Anthon, & Barrett, 2007). In Figure 1B the results for thermally treated pectin at pH 6.3 are presented. It is apparent that with increasing heating time there is an increase in the absorbance at 235 nm and to a lower extent at 280 nm. This increase is mostly due to depolymerization and the formation of β-elimination products that are known to absorb at 235 nm. No large differences with increased processing time in the absorbance at 600 nm can be seen. In the HP/HT treated samples at pH 6.3 (Figure 1D) one can easily see that there is an increase in the absorbance at 235 and 280 nm both with and without the stage of filtration but the increase is much smaller than the increase in absorbance at these wavelengths for only thermally treated samples (for the same treatment time). This is in agreement with the previously published data that for HP/HT treated pectin (pH of 6.5, 110 °C, 600 MPa) β-elimination was stopped after 7 minutes and it was significantly lower than the β-elimination observed at atmospheric pressure at the same temperature (De Roeck et al., 2009). There is also a clear decrease of the absorbance at 600 nm with increasing treatment time for the unfiltered samples that was not observed for the samples that were only thermally treated. Except of some increase in the absorbance of all measured wavelengths after 10 minutes of treatment no change is visible for the UV of the samples treated by HP/HT at the pH of 4.4 (Figure 1C). Important to note that not in all cases the observed changes for the filtered and non-filtered samples are correlated. For example the absorbance at 600 nm for the samples thermally treated at the pH of 4.4 showed a clear decrease without a similar trend observed for the filtered samples.

3.2 The effect of processing on the degree of methoxylation (DM)

The obtained DM of the variously processed pectin samples are presented in Table 2.

Table 2: The measured degrees of methoxylation (DM) of the processed pectin samples with and without a stage of filtration (after processing) at the two pH studied. Temp – Thermal treatment, the value on the right describes the duration (in minutes) of the treatment at 100°C; HPHT –

448 High pressure high temperature, the value on the right describes the duration (in minutes) of the
449 treatment at 100°C and 600MPa.

	pH 6.3		pH 4.4	
Treatment	DM before filtration	DM after filtration	DM before filtration	DM after filtration
Unprocessed	68.41±0.84	68.20±3.14	68.57±2.71	68.71±1.68
Temp 0	62.67±1.22 a	65.42±0.69 a	70.15±3.54 a	72.32±1.25 a
Temp 10	51.77±1.81 b	51.98±0.18 b	71.88±0.34 a	70.21±1.27 a
Temp 20	44.20±0.66 c	45.65±1.35 c	67.74±3.3 a	71.38±1.06 a
Temp 40	38.07±0.52 d	32.51±1.38 d	67.54±3.27 a	70.44±0.29 a
HPHT 0	63.45±0.4 a	63.12±1.04 a	66.01±3.73 a	65.28±0.51 a
HPHT 10	5.85±0.69 b	5.93±0.79 b	53.54±1.17 b	59.91±2.59 a
HPHT 20	7.69±0.45 bc	7.77±1.25 b	49.77±0.17 b	46.4±0.7 b
HPHT 40	4.73±0.03 bd	6.19±0.82 b	50.84±0.34 b	45.53±1.53 b

450 Values are mean ± standard error, mean values in a column with different letters are significantly
451 different at $p<0.05$

452 As can be seen in Table 2 the DM of the samples with no treatment at all (unprocessed) was
453 ~68.5 which is very close to the reported DM of the original pectin by the supplier (70.7,
454 Certificate of Analysis by Sigma Aldrich for lot number 1424480). As expected, a decrease in
455 the DM of the thermally treated samples with increasing treatment time was observed at the pH
456 of 6.3 (Fraeye et al., 2007) while no change in the DM was observed at the pH of 4.4 due to the
457 thermal stability of the pectin at this pH (Axelos & Branger, 1993), although a pervious study at
458 the pH of 4.5 showed a very limited release of methanol even at this acidic pH (Diaz et al.,
459 2007). The rate of the decrease in DM at pH 6.3 was not linear and has decreased with increasing
460 treatment time as expected (Fraeye et al., 2007). The HP/HT treatment at pH 6.3 has led to a very
461 strong and rapid decrease in DM, already after 10 minutes of treatment the DM was 5.85 which
462 is practically a complete demethoxylation. A previous study has shown a very rapid methanol
463 release under HP/HT treatment (De Roeck, Sila, Duvetter, Van Loey, & Hendrickx, 2008) but to
464 the best of our knowledge no study has shown the DM obtained or suggested that already after a
465 few minutes at the pH of 6.3 a complete demethoxylation is observed. At the pH of 4.4 some
466 immediate demethoxylation was observed (from 66 to 53.5 after 10 minutes) but no further
467 demethoxylation with time occurred. No previous reports on the effects of HP/HT on pectin in a

slightly acidic environment are available. The reason for this limited decrease in DM is not completely clear, it is possible that the increased pressure enhances the limited demethoxylation that can occur even at this low pH, similarly to the enhancing effect at higher pH, or it is an artifact of the pH correction to 5. Only upon arriving to the target pressure of 600MPa the pH of the HP/HT sample is expected to reach 4.4, during the pre-heating and the pressure build-up phase the pH is expected to be higher (the pH of the sample was increased to 5 just prior to treatment) which can also increase the demethoxylation rate. The reason for the slightly lower DM at pH 6.3 for the starting conditions at the HP/HT and thermal treatments compared to the unprocessed sample is due to the different definition of the starting point. While the unprocessed sample was not exposed to any heat treatment for the thermally treated condition the starting point is the sample that was allowed to reach 100°C and immediately cooled down. For the HP/HT sample the 0 point was defined as the sample that was allowed to reach 60°C and then immediately cooled down (because 60°C was the starting point for pressure build-up as described at 2.2.3.2). Therefore some demethoxylation during these initial heating stages occurred. Previously, it was suggested that the tendency for aggregation of certain pectins might be due to the presence of some molecules of low DM, although other mechanisms of aggregation such as hydrogen bonds and hydrophobic interactions might also occur (Kravtchenko, Berth, et al., 1992). In our study, no specific trend in DM was observed when comparing identical samples before and after the filtration step, suggesting that variations in DM as such are not the reason for some of the material loss during the filtration. Although it is possible that some limited differences in the DM will not be observed by such comparison because we were unable to directly measure the DM of the pectin remaining on the filter.

3.3 The effect of processing on the formation of reducing ends

The amount of reducing ends formed is a known measure of pectin depolymerization both for acid and alkaline thermally treated samples. The reducing ends formation can also be used to differentiate between the β-elimination and acid hydrolysis. During depolymerization under alkaline conditions the amount of formed unsaturated uronides is correlated to the amount of reducing ends while in acidic condition no unsaturated uronides are formed although there is formation of reducing ends (Diaz et al., 2007).

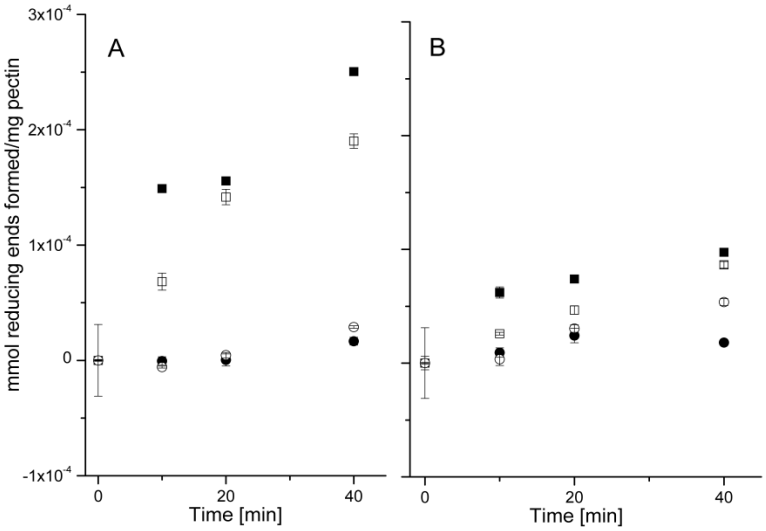

Figure 2: The amount of reducing ends formed for the samples at pH 6.3 (Square) and pH 4.4 (Circle) without (filled shape) and with (empty shape) a stage of filtration. A – Thermal treatment, B – HP/HT treatment.

In Figure 2A one can clearly notice the formation of reducing ends by thermal treatment at pH 6.3. The reducing ends formed are due to the β-elimination process as this increase in reducing ends was also correlated with an increase in the UV absorbance at 235 nm (Figure 1B). A slight formation of reducing ends is also visible at the pH of 4.4 but only after 40 minutes of treatment, this probably correlates to the slight increase in the absorbance at 235 nm (of ~10%) seen in Figure 1A. The formation of reducing ends during HP/HT treatment at pH 6.3 (Figure 2B) was much lower compared to the formation of reducing ends for the thermally treated samples at atmospheric pressure (Figure 2A). This is in line with the lower formation of β-elimination products as is seen by the absorbance at 235 nm in Figure 1D (HP/HT) and Figure 1B (Thermal treatment). The HP/HT treatment also caused some limited increase in the amount of reducing ends under treatment at pH 4.4. The origin of the formed reducing ends is not fully clear, some increase in the absorbance at 235 nm was observed in Figure 1C, but it was very slight. Therefore it is possible that some acid hydrolysis caused the increase in reducing ends. At pH 4.4 in all processes and intensities no clear trend to the effect of filtration appears. On the other hand at pH 6.3 it seems that in most cases the amount of formed reducing ends was larger per mg of

pectin for the unfiltered compared to the filtered samples. This might suggest that the non-filterable aggregates are even slightly more susceptible to the depolymerization than the smaller more filterable pectin although no increase in filterability was observed as will be presented in section 3.4. The reason that in this study we did not compare directly the amount of unsaturated uronides formed to the reducing ends formed (Diaz et al., 2007), to assess the importance of acid hydrolysis in the studied processes is methodological. We believe, as will also be shown in section 3.4, that conformation changes of the UV absorbing molecules linked/interacting with pectin will also occur simultaneously to the formation of unsaturated uronides causing difficulties in result interpretation especially in samples where very little change in the UV absorbance at 235 nm was observed.

3.4 The effect of processing on SEC-UV-MALLS-RI elution profile

Size exclusion multi angle laser light scattering combined with RI and UV detectors can provide an in-depth knowledge regarding the effects of processing on the molecular weight of polymers. In all chromatograms presented in this sub section the concentration signal of the pectin elution profile (calculated from the RI signal using the *dn/dc*) included two main peaks as can be seen for example for the unprocessed pectin samples at both pH values (Figure 3, solid line).

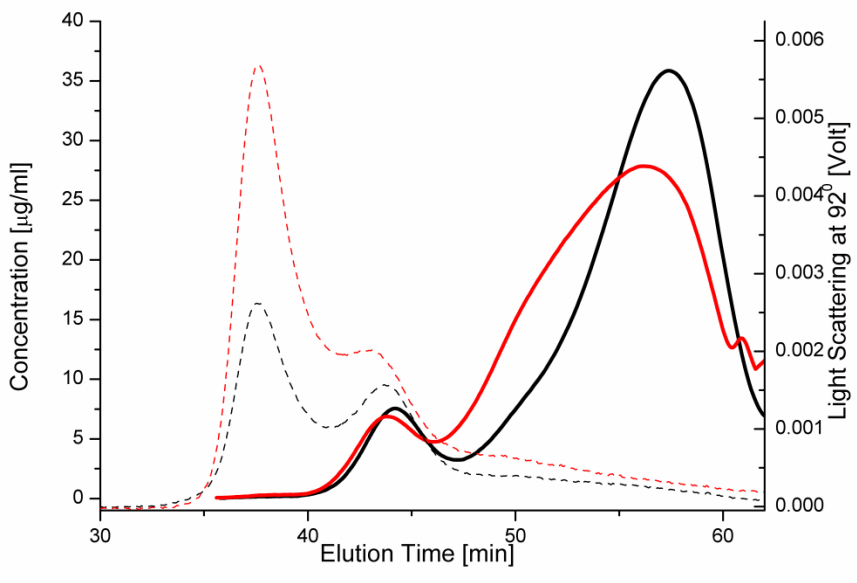

Figure 3: Size exclusion elution profile of unprocessed pectin samples. Concentration chromatogram (solid lines) superimposed with light scattering at 92° angle detector response (dashed lines) for samples at pH 6.3 (red) and 4.4 (black).

In a previous study of a microwave extraction of pectin the presence of a bimodal distribution for pectin on an elution chromatogram was presented and the larger molar mass fraction was suggested to be a compact aggregated network (Fishman, Chau, Hoagland, & Ayyad, 1999). On the other hand a different study has suggested that unlike in industrial lemon pectin in apple pectin a large molecule fraction may consist of individual molecules of very large size (Kravtchenko, Berth, et al., 1992). Important to note that if the samples were not allowed to rest at least overnight after the filtration step and reach equilibrium before injection, the elution chromatogram of the concentration presented only one large peak (not shown). All samples were vigorously shaken for at least 10 minutes after the equilibration to ensure that there is no effect of sedimentation. The first concentration peak with a maximum eluting around 43-44 minutes was in all cases responsible for ~10% of the total mass recovered, while the second one (maximal elution after 56 min) was ~90% of the recovered mass (at both pH conditions), as was determined as area under the curve for the concentration by the software. The light scattering chromatogram (Figure 3, dashed line), that is much more affected by large molecules, constantly presented two peaks: the first that is hardly detectable by the concentration with a maximum around 37.5 minutes is most likely related to very large aggregates with a molecular weight more than $10*10^6$ Da (the limit for separation of the size exclusion columns used) and a second one with a maximum around 43-44 minutes that is related to the peak responsible for 10% of the recovered mass. The peak related to the majority of the mass of the pectins, that is clearly seen as the broad peak of the concentration curve, cannot be clearly seen as a separated peak by the light scattering detector but more as an increase of the intensity over the baseline due to the small size of the polymer combined with the broadness of the peak. The absorption spectra at 235 nm allowed a continuous monitoring of the formation of β-elimination products (for example the elution chromatogram of the thermally treated pectin at pH 6.3, Figure 4B) and could possibly also suggest changes in the conformation or concentration of the co-eluting protein. For the

562 determination of the weight averaged molecular weight (Mw), the elution chromatogram was
563 divided into two regions. The first region was set as the region of the larger particles from the
564 first appearance of a detectable concentration signal up to the minimum between the two peaks
565 visible on the concentration curve (approximately 46-47 minutes). Region two was set from the
566 end of the first region till the beginning of the elution of mono-galacturonic acid that was
567 injected separately (not shown, elution time ~63.5 minutes). The Mw obtained, peak
568 polydispersity and the percentage recoveries compared to the mass before filtration (based on
569 areas of DRI chromatograms (Fishman et al., 2001)) are reported at Table 3.

570 Firstly we would like to address the effect of the elution buffer. In our study it was clear that the
571 obtained pectin Mw is buffer dependent. The difference between the unprocessed samples at pH
572 4.4 and 6.3 are clear both in Figure 3 and in Table 3. The concentration chromatogram of the
573 unprocessed sample at pH 6.3 is shifted to the left compared to an identical sample at the pH of
574 4.4 suggesting higher Mw. Similarly the LS chromatogram of the sample at pH 6.3 is higher,
575 without a significant change in the concentration (Table 3). The obtained calculated Mw are
576 significantly larger at pH 6.3 for the second peak (lower Mw) and are higher (although not
577 significantly) also for the peak with the larger Mw (Table 3). A similar trend was observed for
578 time 0 of thermal and HP/HT treatments, which were only lightly treated. These results are
579 comparable with the results obtained for different citrus pectins that showed lower Mw in
580 LiAc/HAc buffer (pH 4.8) than identical samples in $NaNO_3$ (Fishman et al., 2001) the pH of
581 which is expected to be neutral to slightly acidic. One can explain the differences in the Mw
582 obtained at the two pH values studied, by slightly increased association of several pectin
583 fragments at pH 6.3 that cause the increase in the average Mw for the whole peak. The reason for
584 this increased association is still unclear, although it is possible that some residual Ca2+ (or other
585 divalent ions) present in solution are enough to induce a limited crosslinking between individual
586 pectin molecules through the charged GalA residues even at this relatively high DM. At a lower
587 pH a larger part of the non-methylated GalA is uncharged reducing the possible interaction
588 points. Our preliminary results using inductively coupled plasma mass spectrometry showed that

589 that pectin dialysis (0.5% w/w) against demineralized water resulted in an increase in the levels
590 of Ca^{2+} (from 9.85±0.63 to 77.28±3.54 mg/L**)**. Despite the larger Mw of both fractions at the pH of
591 6.3 it did not seem to have a significant effect on the filterability of the samples. A previous

study has even suggested that aggregated/associated pectin in $NaNO_3$ solution (compared to LiAc/HAc buffer) was actually more soluble (Fishman et al., 2001). The conclusion that can be obtained from these results is that a part of the pectin material in solution, especially for the pH of 6.3 but also possibly to a smaller extent at the pH of 4.4, does not constantly exist as individual pectin molecules but actually at least partially associated with other pectin molecules. This conclusion enhances the importance of taking into account conformation and non-covalent interactions when discussing molecular weight distribution results. Whether this effect is solely due to the pH or due to the different ions used was not evaluated in this work.

3.4.1 Thermally treated pectin

The β-elimination reaction occurring at neutral or alkaline pH was previously shown to reduce the molecular weight of pectins (Diaz et al., 2007). Figure 4 shows the elution profiles of thermally treated pectin samples at pH 6.3.

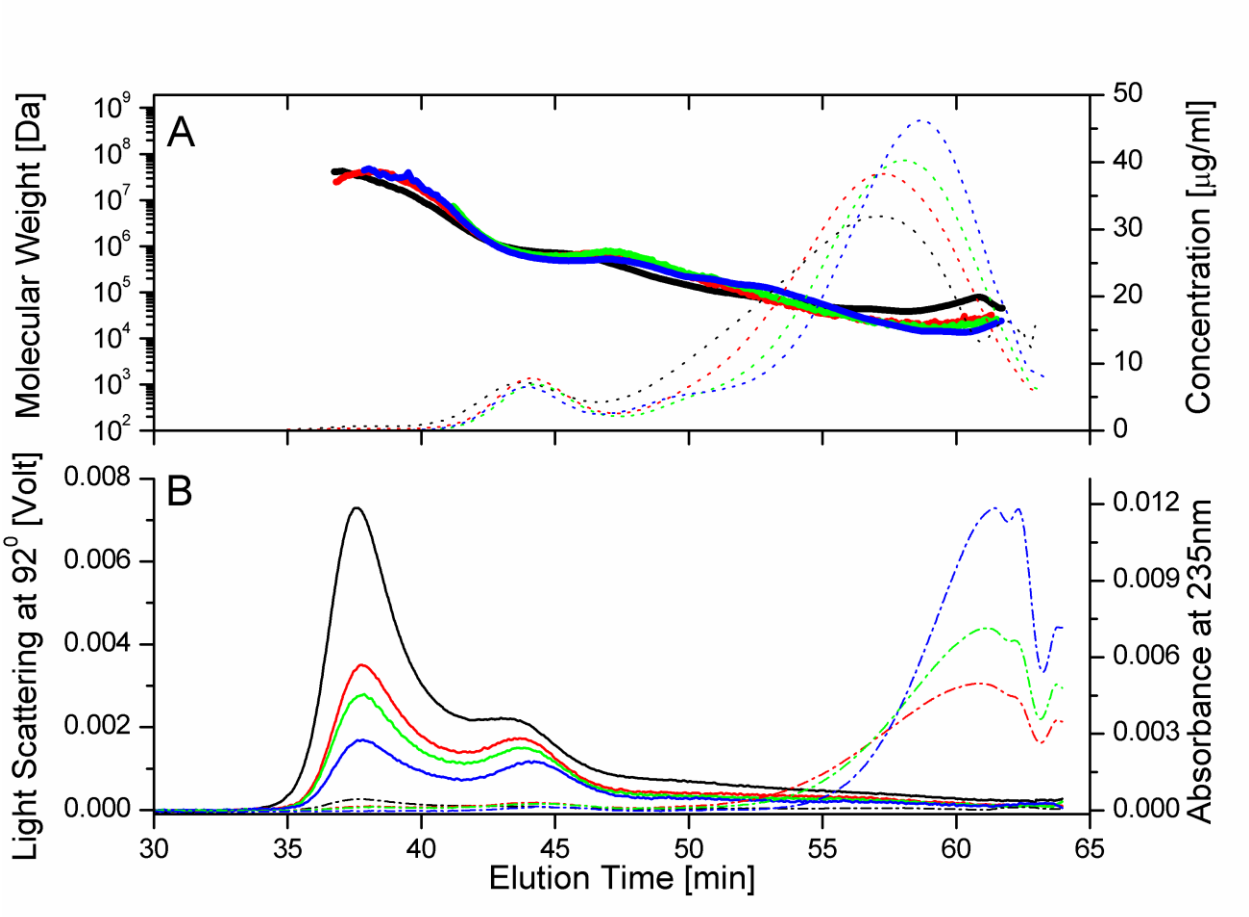

Figure 4: Size exclusion elution profile of thermally treated (100 °C) pectin samples at pH 6.3 (A) Log molecular weight against elution volume (thick solid line) superimposed on concentration chromatogram (dotted curve) (B) Light scattering at 92° angle detector response (solid line) superimposed on the absorbance at 235nm detector response (dot-line-dot). Treatment time: 0 – black; 10 minutes – red; 20 minutes – green; 40 minutes – blue

From the concentration signal it is clear that the thermal treatment caused a shift to the right (the formation of smaller molecules) with increasing treatment time for both peaks. The elution profile of the absorbance at 235 nm is a convenient way to monitor β-elimination and it was clearly seen (Figure 4B) that with increasing treatment time there is an increase in concentration of molecules absorbing at 235 nm, eluting at the end of the concentration chromatogram. To the best of our knowledge this is the first time such method for evaluation of β-elimination is used.

Despite the small shift to the right also for the peak corresponding to the larger molecules (Figure 4A) no appearance of absorbance at 235 nm is visible before 55 minutes of elution, possibly suggesting that the peak around 43 minutes is of some aggregated/entangled pectin rather than of very large individual pectin molecules that would be expected to result, due to the β-elimination in a peak with absorbance at 235nm located just to the right of the original peak similarly to what is observed for the second (smaller) peak. On the other hand heating was reported to dissociate pectin aggregates (Fishman et al., 1999) which in our case would be expected to decrease the fraction of the larger peak. The calculated Mw (Table 3) clearly presents a decrease in Mw with increasing treatment time for both peaks. No change appeared in the percentage of the mass found suggesting that the unfilterable pectin (most probably some very aggregated pectin) did not significantly became more dissociated and more soluble due to the thermal treatment. The polydispersity of the larger peak became smaller while the polydispersity of the smaller peak increased probably due to the formation of a fraction of extremely small depolymerized pectin molecules on the one hand with a small shift of some of the larger molecules to the region of lower molar mass pectins. The results also show that with increasing treatment time, molecules that were already depolymerized by β-elimination can undergo further β-elimination as can be seen by the fact the peak of the absorbance at 235 nm becomes sharper with increased processing time and the tail to smaller elution times (larger molecules) becomes shorter. Additionally, as can be seen in Table 2, the thermal treatment results in an expected decrease in the DM, due to this fact two outcomes can occur that might be superimposed with the effect of the depolymerization on the Mw. The demethoxylation on the one hand results in an increased negative charge for pectin at this pH. Such increased negative charge can cause disassociation of the observed pectins due to electrostatic repulsion. On the other hand if there is sufficient divalent ion concentration (like Ca^{2+}) some cross linking might occur. The fact that there is a continuous decrease in Mw suggests that despite the fact that some traces of Ca^{2+} exist in solution its concentration is not sufficient to induce gelation or even micro-gelation. One cannot exclude the possibility that part of the observed decrease in Mw is caused by disassociation of associated/aggregated individual pectin molecules.

Table 3: The weight average molecular weight (Mw, of the first and second peaks observed at the concentration chromatogram), the percentage of the mass found (relatively to the mass of the

sample prior to filtration), and the polydispersity of the processed pectin samples as calculated from the SEC-MALLS-RI runs. Temp – Thermal treatment, the value on the right describes the duration (minutes) of the treatment at 100°C; HPHT – High pressure high temperature, the value on the right describes the duration (minutes) of the treatment at 100°C and 600MPa.

Treatment	pH	Mw I $*10^6$	Mw II $*10^3$	%Mass found	Mw/Mn I	Mw/Mn II
Unprocessed	4.4	2.47±0.35 a	74.45±4.65 a	0.838±0.003 a	2.752±0.15 a	1.571±0.01 a
Unprocessed	6.3	3.39±0.28 a	99.5±2.5 b	0.827±0.015 a	3.317±0.233 a	1.579±0.05 a
Temp 0	4.4	2.42±0.4 a	79.05±1.65 a	0.838±0.003 a	2.752±0.15 a	1.571±0.01 a
Temp 10	4.4	2.68±0.36 a	79.2±1.4 a	0.83±0.005 a	2.875±0.125 a	1.596±0.015 a
Temp 20	4.4	2.68±0.25 a	77±2.3 ab	0.834±0.004 a	3.014±0.219 a	1.605±0.01 a
Temp 40	4.4	2.51±0.4 a	68.55±1.75 b	0.811±0.013 a	2.936±0.064 a	1.635±0.011 a
Temp 0	6.3	4.38±0.14 a	115.5±12.5 a	0.827±0.029 a	3.334±0.141 a	1.598±0.033 a
Temp 10	6.3	2.43±0.23 b	65.05±1.25 b	0.842±0.005 a	2.823±0.299 a	1.626±0.072 a
Temp 20	6.3	1.13±0.09 c	48.25±1.75 bc	0.812±0.002 a	1.62±0.042 b	1.9±0.051 bc
Temp 40	6.3	1.42±0.08 c	30±1.5 c	0.832±0.003 a	2.546±0.124 a	2.144±0.001 c
HPHT 0	4.4	2.04±0.29 a	55.4±4.5 a	0.765±0.007 a	2.844±0.267 a	1.623±0.032 a
HPHT 10	4.4	5.4±0.37 b	143±12 b	0.829±0.011 b	3.417±0.018 a	1.576±0.019 a
HPHT 20	4.4	7.39±0.19 b	140±1 b	0.778±0.002 a	4.395±0.032 b	1.641±0.045 a
HPHT 40	4.4	7.36±0.54 b	125±4 b	0.824±0.008 b	4.482±0.193 b	1.564±0.008 a
HPHT 0	6.3	3.89±0.31 a	107.5±6.96 a	0.827±0.013 a	3.179±0.141 a	1.535±0.045 a
HPHT 10	6.3	1.76±0.55 b	52.83±10.16 b	0.895±0.005 b	2.176±0.299 b	1.666±0.001 a
HPHT 20	6.3	1.72±0.12 b	47.4±1.2 b	0.805±0.005 a	2.673±0.042 ab	1.63±0.062 a
HPHT 40	6.3	1.27±0.43 b	35.2±11.2 b	0.907±0.008 b	2.343±0.124 ab	1.616±0.016 a

Values are mean ± standard error, mean values in a column for a specific treatment with different letters are significantly different at $p<0.05$.

For the thermally treated pectin at pH 4.4 minimal effects of processing was expected due to the stability of pectins in such pH. Indeed, the concentration curve (Figure 5A) shows only a slight shift to the right and only after 40 minutes of treatment. The molecular weight curves fully overlay on each other and the LS signal is also practically unaffected by the thermal treatment for 10 and 20 minutes, while for the sample treated for 40 minutes there is a small shift to the right for both peaks accompanied with a small decrease in intensity for the peak having a maximum at 44 minutes. At the high elution times a visible increase (although small relatively to the effect at pH 6.3) in the absorbance at 235 nm can be noticed confirming that the observed increase in the absorbance at 235 nm seen for the whole sample in section 3.1 is due to some

depolymerization occurring by β-elimination, and not due to a conformation change of linked UV absorbing molecules. The corresponding decrease in Mw was significant only for the peak of low Mw and only for the 40 minutes treatment. No changes were observed for the percentage of recovered mass. The change in polydispersity of the peak having lower Mw showed a similar trend to the one observed at pH 6.3 although not statistically significant.

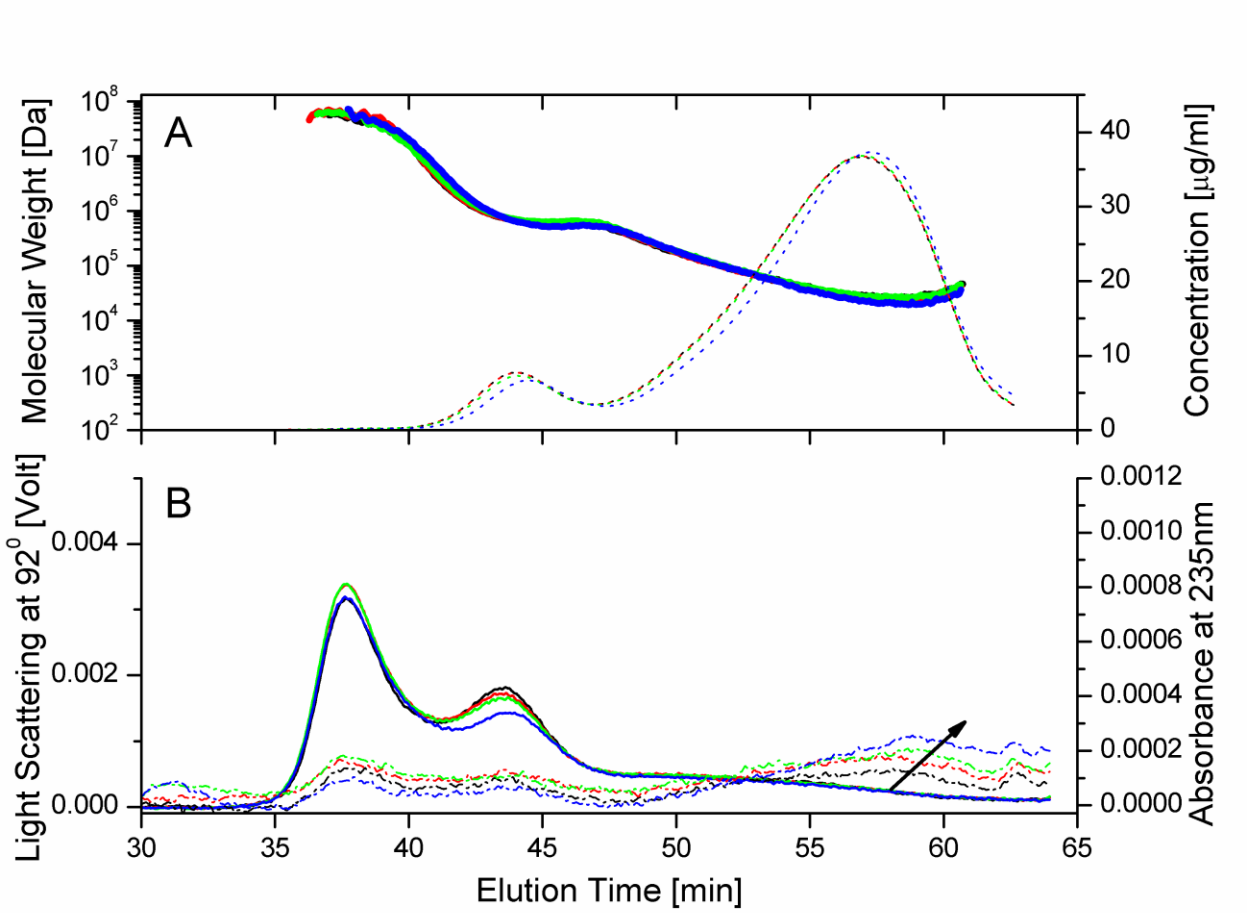

Figure 5: Size exclusion elution profile of thermally treated (100°C) pectin (0.2% w/v) samples at pH 4.4 (A) Log molecular weight against elution volume (thick solid line) superimposed on concentration chromatogram (dotted curve) (B) Light scattering at 92° angle detector response (solid line) superimposed on the absorbance at 235nm detector response (dot-line-dot). Treatment time: 0 – black; 10 minutes – red; 20 minutes – green; 40 minutes – blue. The arrow shows the observed increase in the absorption at 235.

673 The obtained results can also show the limitation of concluding from the results of SEC-MALLS
674 on the original unfiltered systems. Figure 1A clearly shows a decrease in the absorbance at 600
675 nm of the unfiltered systems with increasing temperature. This reduced turbidity can be
676 explained by a significant decrease in particle size, contradictory to the relative stability in Mw
677 observed by SEC-MALLS especially for the larger particles. Most likely the high temperature
678 was able to dissociate large unfilterable aggregates (without increasing their filterability) while
679 very limited effect was induced by the heat treatment on the filterable material.

680 3.4.2 HP/HT treated pectin

681 In Figure 6 the elution chromatograms of the HP/HT treated samples at pH 6.3 are presented. By
682 observing the concentration curve (Figure 6A) no clear trend of a peak shift is noticed.
683 Therefore, by concluding only based on the concentration (RI) chromatogram, one could easily
684 reach the conclusion that HP/HT treatment does not result in significant changes of molecular
685 weight. Similar results (of no peak shift for HP/HT samples compared to visible peak shift for
686 thermally treated samples) were observed for the water soluble fraction of carrots when the
687 elution chromatogram was followed by RI upon thermal treatment (80°C, 0.1MPa) and HP/HT
688 (80°C, 600MPa) (De Roeck et al., 2008). Such conclusion can be reinforced by the limited
689 (compared to the thermal treatment at identical treatment times) increase of the absorbance at
690 235 nm (Figure 1D) suggesting a limited β-elimination process, with no expected acid
691 hydrolysis at this high pH. A limited increase in the absorbance at 235 nm is also visible at the
692 end of the elution chromatogram (Figure 6B). Additionally, as seems from both Figure 1D and
693 from Figure 6B, the β-elimination proceeds although very slowly, even after the DM of the
694 pectin is extremely low (below 10%, Table 2). Another point to be noticed is the decrease in the
695 absorbance at 235 nm at the low elution times (35-45 minutes). This low intensity absorbance,
696 that was correlated with high molecular weight molecules, is seen at both pH values studied and
697 with varying intensities at all treatments. When observing using a diode array the whole
698 collected UV spectrum of an unprocessed pectin sample (pH 6.3) at elution time of 38 and 43
699 minutes (not shown) two distinct peaks with maxima at ~230-240 nm and 280 nm are noticed
700 possibly suggesting that these peak are correlated to protein.

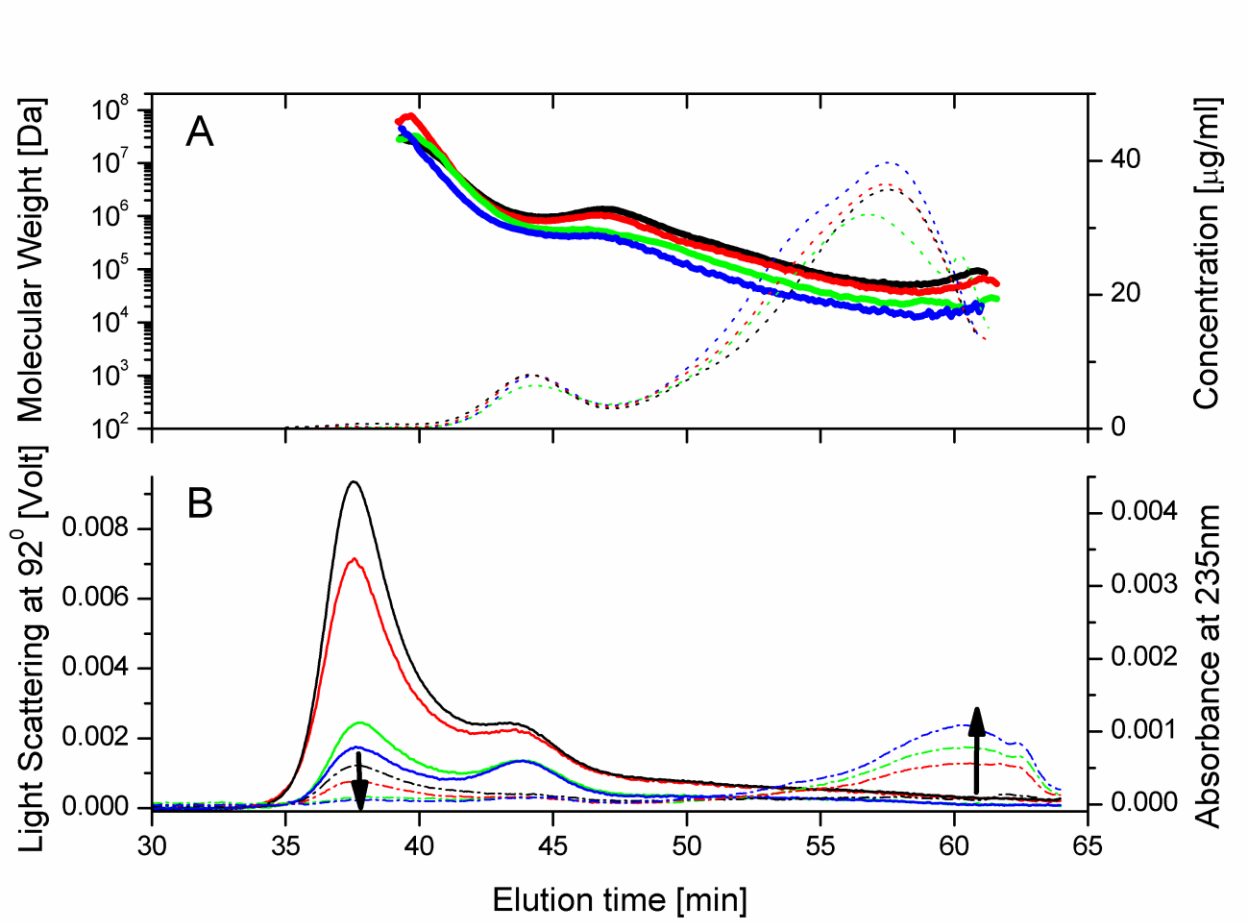

Figure 6: Size exclusion elution profile of High Pressure/High Temperature (100°C, 600MPa) treated pectin (0.2% w/v) samples at pH 6.3 (A) Log molecular weight against elution volume (thick solid line) superimposed on concentration chromatogram (dotted curve) (B) Light scattering at 92° angle detector response (solid line) superimposed on the absorbance at 235 nm detector response (dot-line-dot). Treatment time: 0 – black; 10 minutes – red; 20 minutes – green; 40 minutes – blue. The arrows show the observed changes in the absorption at 235 nm.

When considering also the LS signal (Figure 6B) a very sharp decrease in the LS signal with increasing processing time is observed all over the elution chromatogram. In the calculated molecular weight curve (Figure 6A) one can clearly see that for identical elution time the molecular weight is significantly lower with increasing treatment time. The size exclusion columns separate the molecules based on the hydrodynamic volume and therefore we can

conclude that upon high pressure/high temperature treatment the molecules obtained are less compact. As can be seen in Table 3 the Mw of the pectin samples was significantly reduced upon HP/HT treatment for both peaks. The combination of smaller (in terms of molecular weight) but less compact molecules can explain the lack of the expected, due to the decrease in Mw, shift in the concentration peak (Figure 6A). These results emphasize the necessity of using light scattering in combination with SEC-RI when studying the effects of processing on molecular weight distribution and conformation of pectin. We suggest that the reason for the less compacted conformation of the pectins is the electrostatic repulsion between the negatively charged groups formed upon the total demethoxylation occurring during the treatment. This electrostatic repulsion results in dissociation of individual pectin molecules and thus is responsible for part of the decrease in the observed Mw. This can also explain how despite much smaller formation of reducing ends (compared to the thermal treatment, Figure 2 A and B) in addition to a lower formation of unsaturated uronides (Figure 1 B and D) the obtained Mw for the HP/HT treated pectin is similar to the one obtained after thermal treatment (after 40 minutes of treatment). Additionally, it is clearly seen that most of the decrease in the measured Mw occurred already after 10 minutes of HP/HT treatment similarly to the fact that almost a full demethoxylation was observed already after this treatment time.

Upon HP/HT treatment at pH of 4.4 a reverse trend to the one observed at pH 6.3 is visible. While again practically no shift in the concentration curve (Figure 7A) is visible, the calculated Mw has significantly increased for both peaks as is seen in Table 3. The LS signal (Figure 7B) for the processed pectin is much larger than the LS of the non-treated sample, although in this case it seems that after a maximum at 20 minutes the intensity of the LS signal is decreasing. In contrast to the effect at pH 6.3, at pH 4.4 upon HP/HT treatment there is an increase in the molecular weight at identical elution volumes suggesting a more compact structure. Due to the fact that polymerization of the pectin is not likely and even some depolymerization occurred as can be seen from the limited increase in the amount of reducing ends (Figure 2B) we suggest that association of pectin molecules occurred during the HP/HT treatment that wasn’t dissociated back even after significant equilibration time. The suggested association formed more compact molecules having similar hydration volumes to the untreated sample, therefore no large changes in the elution time maxima are observed. The explanation for this process is not yet clear and

will need further research but we can hypothesize the following mechanism. The DM of the pectin samples only decreased slightly (Table 2) upon treatment, possibly allowing some hydrophobic interactions between the pectin samples. Previously hydrophobic interactions were suggested to be related to pectin crosslinking in cell walls (Sila et al., 2009). The effect of pressure on hydrophobic interactions is a complex phenomenon, as was studied by structural changes of proteins upon pressurization, especially if also elevated temperatures are involved (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998). Generally, it was shown that dissociation of hydrophobic interaction is preferred with increasing pressure. But a recent study suggested that there is a critical pressure above which the molar volume of hydrophobic hydration water becomes larger than the molar volume of free bulk water. At these conditions, the researchers suggested that hydrophobic interaction will be favored over hydrophobic solvation, to minimize the proportion of water involved in the thermodynamically unfavorable hydrophobic hydration. Such pressure based on studies of β-casein was suggested to be around 150 MPa (Huppertz & de Kruif, 2006). In our case we suggest that the high pressure (600 MPa) induced aggregation and compactisation of the pectin molecules stabilized by hydrophobic interactions and possibly other non-covalent interactions. In these samples, similarly to what was observed for the HP/HT treated samples at pH 6.3 a correlation between the change in the intensity of the LS and the absorbance at 235 nm at the low elution times (35-45 minutes) occurs (an increase in the intensity of the LS and an increase in the absorbance at 235 nm).

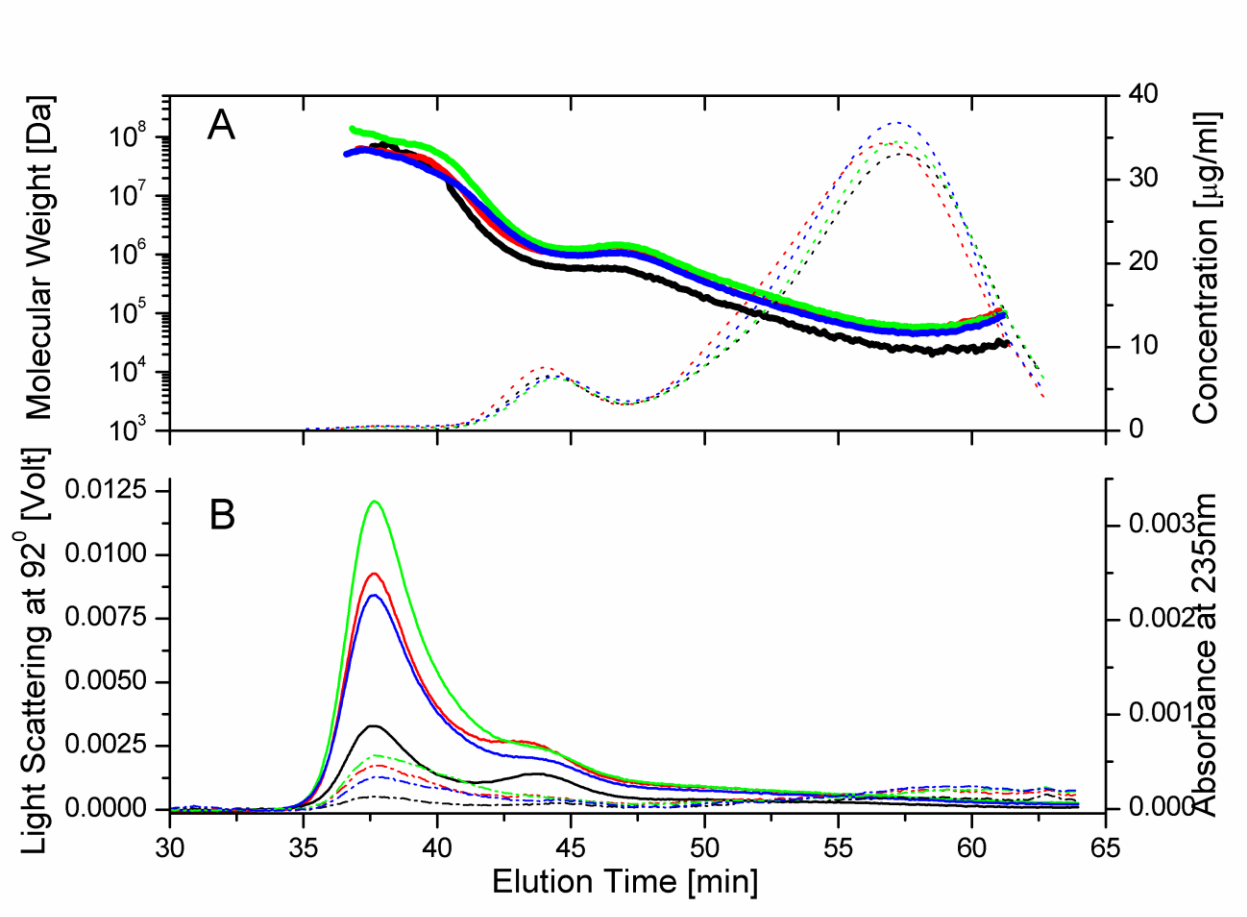

Figure 7: Size exclusion elution profile of High Pressure/High Temperature (100 °C, 600 MPa) treated pectin (0.2% w/v) samples at pH 4.4 (A) Log molecular weight against elution volume (thick solid line) superimposed on concentration chromatogram (dotted curve) (B) Light scattering at 92° angle detector response (solid line) superimposed on the absorbance at 235 nm detector response (dot-line-dot). Treatment time: 0 – black; 10 minutes – red; 20 minutes – green; 40 minutes – blue

4. Conclusions

This work provides for the first time a comprehensive study on the effect of thermal and HP/HT processing treatments on the molecular weight and solution conformation of pectin molecules. The study clearly shows the necessity of combining an absolute molar mass determination to the

SEC-RI method during studies of pectin molecular weight distributions. The results of the thermally treated pectin at both pH and comparison with previously published data show that SEC-MALLS-RI-UV is a reliable and stable method capable of providing valuable data regarding changes in pectin due to processing. The fact that at least at a near to neutral pH some association of pectin molecules exists requires taking this fact into account when interpreting obtained molecular weight data and when considering the solution behavior of pectin. The static high pressure treatment combined with high temperature induced a decrease in the Mw of the sample at pH 6.3 while increased the observed Mw of the samples at pH 4.4, partially due to differences in pectin association at these pH values. While filtration prior to injection into the SEC-MALLS is necessary, the observed loss of material can cause in some cases contradicting results with the original unfiltered sample. For all the different systems and treatments it seems that the change (or absence of change) in the absorbance intensity at 235 nm in the region of the larger particles (elution time 35-45min) is well correlated with the overall observed increase in molecular weight. The reason for this is still unclear but it might be related to conformational changes of pectin linked proteins or their surroundings. It is also not known whether the changes in conformation are only probed by the protein or that the processing changes the protein-pectins interaction.

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