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# Influence of enzymatic and acidic demethoxylation on structure formation in sugar containing citrus pectin gels

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**Influence of enzymatic and acidic demethoxylation on structure formation in sugar containing citrus pectin gels**

H. Kastner*, U. Einhorn-Stoll, S. Drusch

Technische Universität Berlin, Food Technology and Food Material Science, Königin-Luise-Strasse 22, D-14195 Berlin, Germany

* Corresponding author; e-mail-address: h.kastner@tu-berlin.de

*Abstract*

This paper presents the impact of different pectin demethoxylation methods and of their side effects on the molecular properties of the resulting pectin samples and on the structure formation in model gels comparable to typical industrial applications.

A high-methoxylated citrus pectin (HMP) was demethoxylated using hydrochloric acid or pectin methylesterases of plant (pPME) or fungal (fPME) origin. pPME treatment causes a more block-wise distribution of free carboxyl groups, fPME or acidic treatment a random distribution. Twelve pectin samples with four different degrees of methoxylation (DM) between 62% and 41% were prepared. Their gelation process was studied by oscillatory measurements.

Pectin samples from pPME treatment started structure formation at higher temperature and the final gels were more elastic in comparison to pectin from the two other modification types. The impact of the block-wise distribution of the increasing number of free carboxyl groups became more evident with decreasing DM. The gelling process of pectin samples with random distribution was comparable at any DM.

Side effects of all demethoxylation reactions were an altered sodium ion content (high in enzymatically treated pectin, close to zero in acidic treated) and a different molecular weight (decreased with increasing demethoxylation). These side effects additionally altered the gelation process and the final gel properties in different ways.

## 1. Introduction

Pectin has a broad range of application in the food industry as thickener, gelling agent and stabilizer due to the ability of network formation under various environmental conditions. Pectin is extracted from different botanical sources using acids (May, 1990; Rolin, 2002), and raw material as well as extraction conditions determine the pectin quality. After extraction, commercial pectin is often amidated by alkaline or demethoxylated by enzymatic procedures. Differences in the processing conditions alter the chemical characteristic of the pectin (see "Theoretical background" below) and, thus, affect the structure formation of their gels. Demethoxylation is one of the main modification processes. It is performed by chemical or enzymatic methods, which have different side effects. For instance, acidic treatment removes neutral sugar side chains as well as sodium ions from the pectin molecule, whereas enzymatic modification using pectin methylesterase does not affect neutral sugars but adds sodium ions by using NaOH to keep pH constant. These ions bind strongly to the newly formed free carboxyl groups (Einhorn-Stoll, Kastner, Urbisch, Kroh, & Drusch, 2018) and influence some pectin properties.

Pectin gelation has been widely investigated (i.e. Christiaens, Buggenhout, et al., 2016; Lopes da Silva & Rao, 2007; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995; Yapo & Gnakri, 2015). It is challenging to compare the results of the studies due to differing botanical origin and varying content of the pectin, model gel composition, gel cooling conditions or measured structuring parameters. Some authors prepared their pectin samples under defined conditions, whereas others used commercial pectin without knowing or considering the production conditions. The impact of preparation and modification conditions on pectin gels in a sugar-acid environment has not been investigated in detail up to now. Previous studies of our group (Kastner, Einhorn-Stoll, & Drusch, 2017; Kastner, Einhorn-Stoll, & Senge, 2012; Kastner et al., 2014) have shown that citrus pectin samples with similar chemical characteristics, such as degree of methoxylation (DM) and molecular weight, differed in their gelling process and final gel properties. This was found for both HMP and LMP gels. The results were, however, inconsistent, what was attributed to the different origins and unknown processing conditions of the tested commercial pectin samples.

The presented study is the first systematic investigation of the impact of acidic and enzymatic demethoxylation methods on the structuring process of the modified pectin samples in high-sugar gel systems. Groups of pectin samples with similar degree of methoxylation but varying distribution of free carboxyl groups were prepared from a commercial citrus pectin under defined conditions. The structuring process and the final gels were studied at different levels of DM by small deformation oscillatory rheological tests. It was expected that the varying pattern of free carboxyl groups, resulting from the different methods, would have the dominating impact on the pectin gelation. Moreover, an additional influence of side effects of the demethoxylation process, in

particular depolymerisation and increase or decrease of the content of sodium ions, on pectin gelation was assumed and examined in detail.

*Theoretical background*

Pectin is a complex polysaccharide and major component of the cell-wall of plants. Depending on botanical origin, development stage of the plant tissue and extraction and modification methods, the chemical characteristic of pectin differs strongly. The pectin polymer contains a high share of $\alpha$-(1,4)-linked D-galacturonic acid residues (GalA). They form linear homogalacturonan sections and, together with rhamnose, branched rhamno-galacturonan sections (Voragen, Coenen, Verhoef, & Schols, 2009). Moreover, the GalA residues at C-6 position can be methylesterified or amidated. At C-2 or C-3 position, the GalA may be acetylated as e.g. in sugar beet pectin (Ridley, O'Neill, & Mohnen, 2001; Willats et al., 2001).

Depending on the degree of methoxylation (DM), pectin is classified as high-methoxyl pectin (HMP, 50% or above of carboxyl groups esterified) and low-methoxyl pectin (LMP, less than 50% of carboxyl groups esterified) (Lopes da Silva & Rao, 2007). The non-esterified (free) carboxyl groups are distributed along the pectin backbone either randomly or block-wise. The degree of blockiness (DB) describes this distribution pattern. It indicates the ratio of non-esterified mono-, di- and trimers of galacturonic acids, cleaved by endopolygalacturonase, to the total amount of non-esterified galacturonic acids. Daas et al. (1998) introduced the DB, and the impact on pectin gelation was investigated and discussed by several researchers (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Limberg et al., 2000b; Ngouémazong, Tengweh, et al., 2012). The determination of the DB is a complex procedure. Therefore, Glahn and Rolin (1996) suggested the screening method of testing the calcium sensitivity (CS), providing information on calcium reactivity of pectin and, indirectly, on the pattern of free carboxyl groups. Dissolved pectin with a mainly block-wise pattern has a higher ability to interact via calcium bridges, therefore the viscosity of this solution is higher than that of pectin with a random pattern.

Pectin with a defined DM and pattern of free carboxyl groups along the backbone is designed by modification procedures using chemical treatment or pectin methylesterase (PME). Depending on the origin of the PME, different patterns of free carboxyl groups are achieved, a more block-wise pattern with PME of plant origin, or a more random pattern using PME of fungal origin (Fraeye, Colle, et al., 2010; Limberg et al., 2000a; Ralet, Crépeau, Buchholt, & Thibault, 2003). Any acidic or alkaline chemical demethoxylation of pectin initially results in a random distribution of the free carboxyl groups (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010; Thibault & Ralet, 2003). With decreasing DM, however, also these pectin form blocks of free carboxyl groups. These blocks are necessary for the formation of stable egg-box junction zones with calcium, requiring at least blocks of eight to fourteen consecutive free carboxyl groups (Powell, Morris, Gidley, & Rees, 1982;

Liners, Thibault, & Cutsem, 1992; Luzio & Cameron, 2008; Powell et al., 1982), would not be possible in these LMP.

DM and pattern of free carboxyl groups strongly determine the functional properties of pectin, and even small differences in the molecular structure of modified pectin has a strong effect. The most important property of pectin, the ability to form gels, is additionally influenced by environmental factors such as added ions (especially calcium), pH (degree of dissociation of the free carboxyl groups) and added sugars. The general impact of these factors on pectin gelation mechanisms is described in several references (Burey, Bhandari, Howes, & Gidley, 2008; Fraeye, Duvetter, et al., 2010; Jensen, Rolin, & Ipsen, 2010; Lopes da Silva, Gonçalves, & Rao, 1995; Thakur, Singh, Handa, & Rao, 1997), as follows:

- Pectin with high DM and low DB (mainly random distribution of the carboxyl groups) forms gel via hydrophobic and hydrogen bonds by cold-set gelation in an environment containing sugar and acid at pH < 3.5, corresponding to the $pK_a$ of pectin.
- Pectin with low DM forms additional bonds via calcium bridges by ionotropic gelation due to the partly block-wise distribution of the free carboxyl groups. This mechanism requires none or less sugar and acid and is possible also at pH > 3.5.
- Pectin with high DM and high DB (partly block-wise distribution of the methoxyl groups) is to a certain extent able to undergo ionotropic gelation, too. It forms gels at pH > 3.5 and is applied in acidified dairy drinks with pH up to 4.5.

Detailed investigations of the impact of the pattern of free carboxyl groups on pectin gelation gave varying results. For instance, Rolin & Vries (1990) showed that HMP with a block-wise distribution started to gel at higher temperatures than HMP with a more random distribution. Löfgren et al. (2005) obtained the shortest gelation time for pectin samples with high DB without adding calcium ions. They also reported, that two gels containing 60% sucrose and pectin with varying pattern of free carboxyl groups had completely different properties when calcium was added, and that the pH was changed. Ström et al., (2007) showed, that pectin gel properties did not necessarily correlate with DB. Ngouémazong, Jolie, et al. (2012) investigated the stiffness of pectin gels and concluded, that during ionotropic gelation junction zones of pectin with rather random pattern might be shorter but their number was higher, compared to a pectin with more block-wise pattern at same DM.

Though the impact of DM and pattern of free carboxyl groups on pectin gelation and gel properties has been widely examined, the results are not consistent and only partly comparable, and several details require further investigation.

## 2. Materials and methods

### 2.1 Pectin samples

High methoxylated citrus pectin (not standardized) with a DM of 68% (named as OP68) was obtained from CP Kelco (Lille Skensved, Denmark). It was modified enzymatically by fungal PME (f-pectin) and plant PME (p-pectin), as well as chemically by acidic de-esterification (a-pectin). Each modification type was used to prepare four levels of demethoxylation (approximately 62%, 57%, 50% and 41% DM), resulting in 12 different pectin samples. The physio-chemical properties of the 57% and 41% DM pectin samples were characterized already in a previous study (Einhorn-Stoll, Kastner, Hecht, Zimathies, & Drusch, 2015). All chemicals used were of analytical grade.

### 2.2 Enzymes

The two enzymes used in the study were fungal PME (fPME, Fructozym Flot from *Aspergillus niger*, Erbslöh, Geisenheim, Germany) and plant PME (pPME) from orange peel, prepared in the laboratory according to the methods of Arbaisah, Asbi, Junainah, & Jamilah (1997) and Kim, Teng, & Wicker (2005). Oranges were purchased from the local supermarket.

### 2.3 Demethoxylation of the pectin

The procedure for enzymatic demethoxylation was based on the methods of Williams, Foster, & Schols (2003) and Limberg et al. (2000a), and was performed by the pH-stat-method using a Titrando with 50-mL dosing unit (902 Titrando and 800 Dosino; Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany). The pH of the pectin solution (1 wt. %) was kept constant with 0.25 M NaOH at the optimum pH of the PME (7.4 for pPME and 4.4 for fPME, respectively). To stop the process at the intended DM and to inactivate the enzyme, the pH of the solution was decreased to 3.2 and the solution was then heated at 90 °C for 10 min.

Chemical demethoxylation was performed by acidic modification at room temperature. The OP68 (1 wt.%) was dissolved in 0.5 M or 2 M hydrochloric acid solution for HMP and LMP samples, respectively, as described by Einhorn-Stoll, Glasenapp, & Kunzek (1996). After demethoxylation, all pectin samples were precipitated by adding the fourfold volume of 95 vol% ethanol. The precipitate was washed at least five times with 95 vol% ethanol, coarsely ground, dried at 50 °C for at least 3 h and milled (ZM1 with 250 µm sieve, Retsch, Haan, Germany). The samples were stored at -10 °C until further application.

### 2.4 Analytical characterization of model pectin samples

Degree of methoxylation and galacturonic acid content (GC) were analyzed photometrically. The chromotropic method of Bäuerle, Otterbach, Gierschner, & Baumann (1977) was used for DM and

the hydroxydiphenyl method of Blumenkranz & Asboe-Hansen (1973) for the determination of the free uronic acids.

The intrinsic viscosity ([η]) of pectin solutions was analyzed using a LOVIS rolling ball viscometer (LOVIS 2000M; Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with a 1.59 mm capillary and a steel ball (d = 1.5 mm). Pectin samples were solubilized for 24h in sodium oxalate buffer (0.15 M sodium chloride and 0.005 M sodium oxalate, pH was adjusted to 6.0 using 0.1 M sodium chloride) at concentrations of 0.2, 0.1, 0.05, 0.025, 0.0125% (w/v). Pectin solutions and solvent were investigated at 20 °C and at an angle of 50°. The density of pectin solutions and solvent were measured using a density meter DMA38 (Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) at 20 °C. The relative ($\eta_{rel}$) and the specific ($\eta_{sp}$) viscosities were calculated from the following relations (Harding, 1997; Morris, Foster, & Harding, 2002):

$$\frac{\eta}{\eta_0} = \left(\frac{t}{t_0}\right)\left(\frac{\rho}{\rho_0}\right) = \eta_{rel} \tag{1}$$

where $\eta$ is the viscosity, $t$ is the efflux time and $\rho$ is the density of the pectin solution as well as $\eta_0$ is the viscosity of the solvent, $t_0$ and $\rho_0$ are the corresponding efflux time and density, respectively. The specific viscosity is defined as:

$$\eta_{sp} = \eta_{rel} - 1 \tag{2}$$

Extrapolating the specific viscosity to zero concentration, the intrinsic viscosity was obtained (Chou & Kokini, 1987):

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} \tag{3}$$

where $c$ is the concentration of the pectin solution. The [η] is related to the molecular weight by an empirical relationship, the Mark-Houwink equation (Houwink, 1940). The molecular weight (M) of pectin samples was determined by:

$$[\eta] = kM^{\alpha} \tag{4}$$

using values for the coefficients: $\alpha$ (0.79) and $k$ (2.16 x $10^{-2}$), which are suitable for pectin (Berth, Anger, & Linow, 1977; Tillmann Schmelter, Wientjes, Vreeker, & Klaffke, 2002).

The molecular weight distribution of the pectin was determined by gel permeation chromatography (GPC) using the parameters and instruments as described by Wegener, Kaufmann and Kroh (2017). The calibration function was as follows: elution time 15 min ≈ 380 kDa, 17 min ≈ 100 kDa, 19 min ≈ 12 kDa and 21min ≈ 0.3 kDa (Einhorn-Stoll et al., 2018).

The ash content was determined in a muffle furnace at 525 °C. The ash was used to measure the calcium content of the OP86 and sodium content of all pectin samples with a flame photometer (Jenway PFP7, Jenway, Staffordshire, USA) as described by Vetter & Kunzek (2003).

The calcium sensitivity of HMP was determined according to Glahn & Rolin (1996). The measurements were carried out using a Brookfield rotational viscometer, type DV-II+Pro LV (AMETEK GmbH - BU Brookfield, Lorch, Germany) at 20 °C using the spindle V72 at 40 rpm. The calcium sensitivity was calculated for a pectin sample as the difference in viscosity between the sample with ($\eta_{Ca2+}$) and without calcium ($\eta_0$):

$$CS = \eta_{Ca^{2+}} - \eta_0 \tag{5}$$

*2.5 Rheological properties and structuring parameters*

Gels were prepared in triplicate, using the HMP method (sugar-acid environment) for the pectin samples with 62% and 57% DM and the LMP method (sugar-calcium environment) for the samples with 50% and 41% DM. The method for HMP gels was described in detail in Kastner et al. (2014): 2.75 g pectin (0.27 wt.%) was dissolved in 430 g demineralized water and 647.3 g sucrose was added. The total mass was reduced to 1020 g by boiling. Afterwards, 7 mL of 48.8% w/v tartaric acid solution was added. The final solutions had a pH of 2.2 and were within 64.5 – 65.5 wt.% total solids.

The method for LMP gels was described before (Kastner et al., 2012): 6 g pectin (0.67 wt.%) and 264 g sucrose were dissolved in 637.5 g demineralized water, 7.5 mL 54.3% w/v citric acid solution, 15 mL 6% w/v sodium citrate solution and 37.5 mL 2.205% w/v $CaCl_2$ solution. The solution was reduced by boiling to a final mass of 900 g. The final solutions had a pH of 2.8 and a total solids content of approximately 32 wt.%. The total solids contents for HMP and LMP gels were determined by an automatic refractometer (Schmidt and Haensch, Berlin, Germany).

The viscoelastic behavior of the samples during cooling was characterized by small deformation, oscillation measurements using a rheometer (Physica MCR 301, Anton Paar, Ostfildern, Germany) equipped with a profiled rotational cylinder (CC27/P1, diameter 26.66 mm, length 40.01 mm) and a Peltier cylinder temperature system (TEZ 150P). Samples were transferred into the pre-heated rheometer (105 °C) and cooled to 10 °C at a standard cooling rate of 1 K/min. The dynamic rheological parameters storage modulus (G') and loss modulus (G'') were recorded during cooling at constant frequency of 1 Hz and strain of $10^{-3}$ as previously described (Kastner et al., 2012, 2014). The final structure of the gels was characterized by the loss factor ($\tan\delta_{end}$) after cooling at 10 °C.

To describe the gelling process, the following structure parameters were calculated: the classical gel point (GP) was determined as the cross-over of G' and G'' ($\tan\delta$ = G''/G' = 1), and the initial structuring temperature (IST) and the critical structuring temperature (CST) were calculated from the structuring velocity curve (dG'/dt) using OriginPro9.1 software (OriginLab Corp., Northampton, USA). IST is the temperature at which the value dG'/dt differed from zero for the first time, and CST

is the extrapolated temperature of the first strong increase of dG'/dt (Kastner et al., 2012). The presented structuring velocity curves were the average of at least 3 measurements.

*2.6 Statistical analysis*

In order to determine the differences of the individual samples in relation to the DM, a one factorial ANOVA followed by a post hoc test (Tukey) was applied, after a normality test (Shapiro-Wilk). To analyze the effect of pectin modification on structuring temperatures and final gel properties, the non-parametric Kruskal-Wallis test was used. All tests were performed with OriginPro9.1 software (OriginLab Corp., Northampton, USA) at a significance level of 0.05 (95% confidence interval).

## 3. Results

### 3.1 Pectin characterization

Three types of modified citrus pectin, differing in the DM as well as in the distribution of free carboxyl groups along the backbone, have been produced by enzymatic and chemical treatments. The molecular parameters of all samples are shown in Table 1. Demethoxylation of commercial high-methoxylated citrus pectin (OP68, DM of 68%) by pPME (p-pectin), fPME (f-pectin) and acidic (a-pectin) treatment resulted in samples with an average DM of 62%, 57%, 50%, 41%, respectively. Therefore, the groups were named for HMP as DM62 and DM57, as well as for LMP as DM50 and DM41. The DM of the pectin samples within the single groups in general differed not significantly, despite of the samples of DM50: The DM of P51 and F49 differed significantly, but those of P51 and A50 as well as those of F49 and A50 were not significantly different (Table 1).

Table 1  Molecular parameters of reference sample and modified samples in dependence on the method of demethoxylation (pPME, fPME and acidic treated pectin samples). Degree of methoxylation (DM), galacturonic acid (GC), intrinsic viscosity ([η]) with the fit of the curve, molecular weight (MW) and sodium ion content (Na$^+$).

| | Sample | DM* (%) | | GC (%) | | [η] (cm$^3$/g) | | MW (kDa) | Na$^+$ (mg/g) | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Reference* | OP68 | 68.0$^a$ | ± 0.9 | 83.1 ± 1.5 | | 538 ± | 10 | 367.3 | 10.5 ± | 0.0 |
| *pPME* | P61 | 60.9$^b$ | ± 0.5 | 84.7 ± 1.3 | | 528 ± | 8 | 358.6 | 16.6 ± | 0.5 |
| | P57 | 56.8$^c$ | ± 0.6 | 86.5 ± 0.8 | | 503 ± | 5 | 337.3 | 21.4 ± | 0.1 |
| | P51 | 51.4$^d$ | ± 0.6 | 82.1 ± 0.4 | | 320 ± | 6 | 190.3 | 20.2 ± | 0.0 |
| | P40 | 40.4$^f$ | ± 0.6 | 84.9 ± 1.1 | | 239 ± | 8 | 131.5 | 25.1 ± | 0.1 |
| *fPME* | F62 | 61.9$^b$ | ± 0.3 | 88.5 ± 1.0 | | 531 ± | 9 | 361.2 | 11.0 ± | 0.1 |
| | F56 | 56.1$^c$ | ± 0.6 | 94.2 ± 2.0 | | 530 ± | 7 | 360.4 | 12.6 ± | 0.1 |
| | F49 | 49.0$^e$ | ± 0.2 | 82.3 ± 0.4 | | 309 ± | 9 | 182.0 | 20.8 ± | 0.1 |
| | F42 | 41.9$^f$ | ± 0.5 | 78.0 ± 0.1 | | 298 ± | 8 | 173.9 | 24.3 ± | 0.4 |
| *Acidic* | A62 | 62.0$^b$ | ± 0.1 | 90.0 ± 0.6 | | 470 ± | 6 | 309.5 | 0.7 ± | 0.0 |
| | A57 | 57.3$^c$ | ± 0.3 | 93.9 ± 1.2 | | 453 ± | 7 | 295.4 | 0.5 ± | 0.0 |
| | A50 | 49.7$^{d,e}$ | ± 0.7 | 88.3 ± 0.4 | | 421 ± | 3 | 269.3 | 0.0 ± | 0.0 |
| | A42 | 41.5$^f$ | ± 0.7 | 81.7 ± 1.0 | | 414 ± | 4 | 263.6 | 0.1 ± | 0.0 |

* Different letters denote significant differences between individual samples as found by one factorial ANOVA followed by post hoc test (Tukey)

Results in Table 1 show, how the intrinsic viscosity decreased with increasing demethoxylation. The intrinsic viscosity at the lowest DM (DM41) was reduced to about 44% (239 cm$^3$/g) of the value of OP68 (538 cm$^3$/g) for p-pectin, to 55% (298 cm$^3$/g) for f-pectin and to 67% (414 cm$^3$/g) for a-pectin (Table 1). The intrinsic viscosity of enzymatically and acidic treated samples differed at most DM. At DM well above 50%, the a-pectin samples were more depolymerized (A57 = 453 cm$^3$/g) than the enzymatically treated (P57 = 503 cm$^3$/g and F56 = 530 cm$^3$/g). However, at DM around and below 50%, the enzymatically treated samples were more depolymerized (e.g. P51 = 320 cm$^3$/g and F49 = 309 cm$^3$/g) than the acid treated samples (e.g. A50 = 421 cm$^3$/g). These results of intrinsic viscosity are supported by GPC data (Fig. 1). The samples with lower DM (DM41) showed a broader

molecular weight (MW) distribution (elution time 12-17 min) than samples with DM57 (elution time 12-16 min) around an MW of 380 kDa (elution time 15 min). With further demethoxylation, the main peak decreased, and a slight additional peak was formed with a MW of about 12 kDa (elution time 19 min) for the DM41 group.
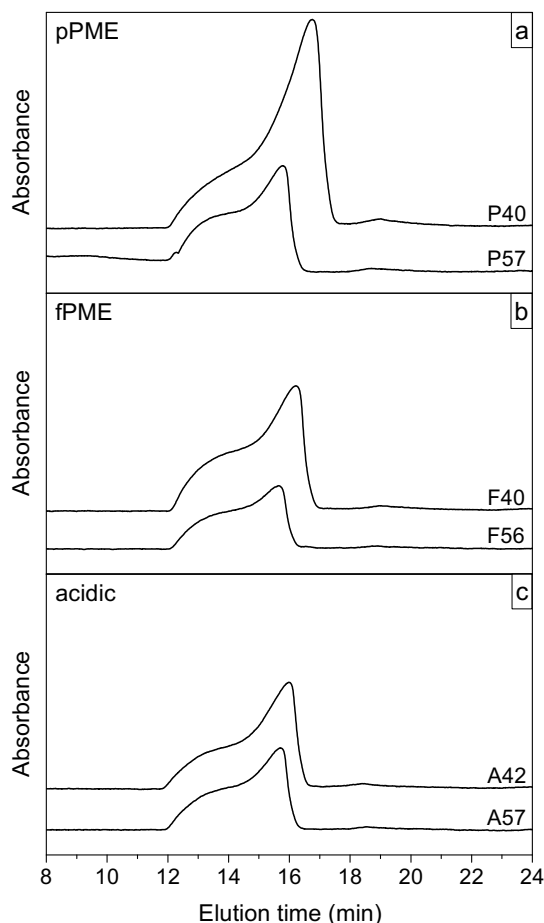


Fig. 1    *GPC images of modified pectin samples, treated by (a) plant PME, (b) fungal PME or (c) acid. HMP group with average DM of 57% (P57, F56 and A57) as well as LMP group with average DM of 41% (P40, F40, A42).*

Apart from demethoxylation and depolymerisation, all modifications altered the sodium content of the pectin samples: It became higher in the p-pectin (16.6 - 25.1 mg/g) and f-pectin samples (11.0 - 24.3 mg/g) than in OP68 (10.5 mg/g), but it was close to zero in the a-pectin samples (0.7 - 0.1 mg/g) (Table 1). Comparing the enzymatically treated samples, the pPME treated HMP samples (16.6 and 21.4 mg/g) had a higher sodium content than the fPME treated (11.0 and 12.6 mg/g), whereas for the LMP the sodium contents of the corresponding p- and f-pectin samples were similar (Table 1).

The different modification methods determined the more block-wise or random pattern of free carboxyl groups of the HMP samples, as reflected by the calcium sensitivity (Table 2). The values of the p-pectin samples DM62 and DM57 were about 100x higher (427.2 and 509.0 mPas, respectively) than the corresponding values of f-pectin and a-pectin samples (4 to 6 mPas).

Table 2  Comparison of calcium sensitivity (CS) of high-methoxylated samples, treated by pPME (P), fPME (F) and with acid (A).

|  | Sample | CS (mPas) |
|---|---|---|
|  | P61 | 427.2 |
| DM62 | F62 | 4.0 |
|  | A62 | 5.9 |
|  | P57 | 509.0 |
| DM57 | F56 | 4.0 |
|  | A57 | 5.2 |

## 3.2 Rheological characterization of the pectin gelation

### 3.2.1 Structure formation and structuring temperatures

The structuring velocity (dG´/dt) of the HMP samples is shown in Fig. 2a. The start of the gelling process (IST) of these samples differed significantly. At DM62, the order of nearly all structuring temperatures (IST, CST and GP) was p-pectin > f-pectin > a-pectin samples (Fig. 3a). At DM57, the structuring temperatures for p-pectin were considerably higher than those of f-pectin and a-pectin, which had similar IST, CST and GP.
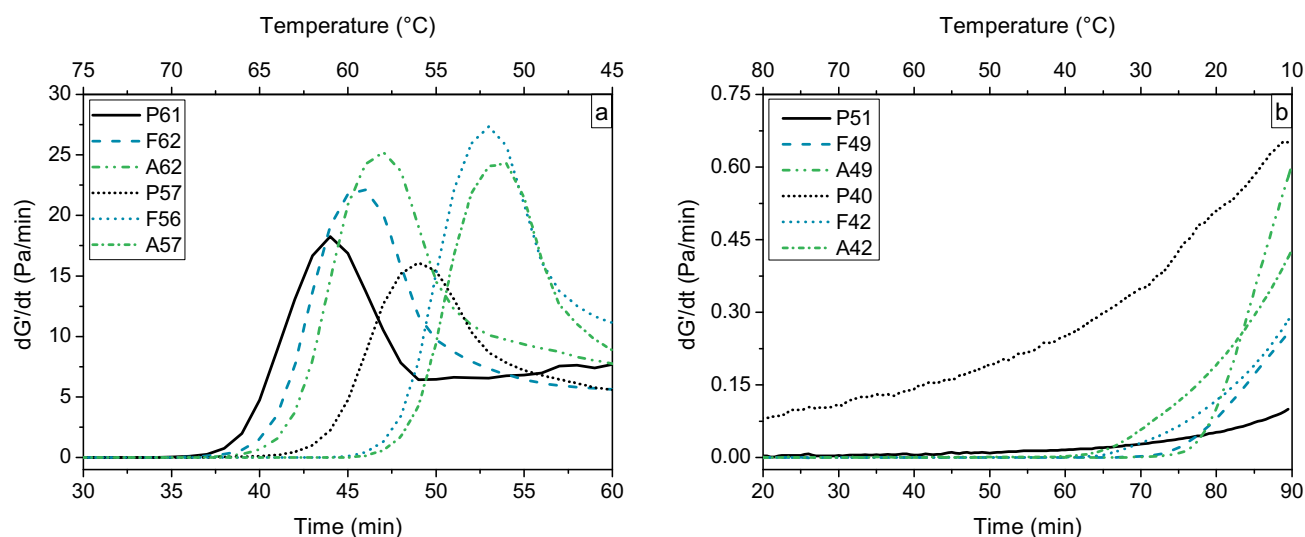


Fig. 2   Influence of pectin demethoxylation method on the structuring velocities (dG´/dt, mean curves) during cooling at 1 K/min. Sugar-acid gels of DM62 and DM57 samples (HMP) during 75 to 45 °C in (a) and sugar-calcium gels of DM50 and DM42 samples (LMP) during 80 to 10 °C in (b).

In general, the structuring velocity of all gels increased during cooling, and was higher for sugar-acid gels of HMP than for calcium gels of LMP (Fig. 2b). The structure formation of the DM50

samples started later and at lower temperature than that of the DM41 samples. The latter, however, showed a slower rate of increase in structuring velocity.

The structuring temperature of the calcium gels increased with decreasing DM (Fig. 3b). The calcium gels were cooled down to 5 °C, because up to 10 °C no GP of the LMP samples with a random free carboxyl group distribution were identified. Thus, the determined gel points were 8 °C for F49, 11 °C for F42, 6 °C for A50 as well as for A42. No sol-gel transition was detected for the p-pectin samples; at the beginning of rheological measurements, the gels underwent pre-gelation with elastic properties dominating over viscous properties (data not shown).
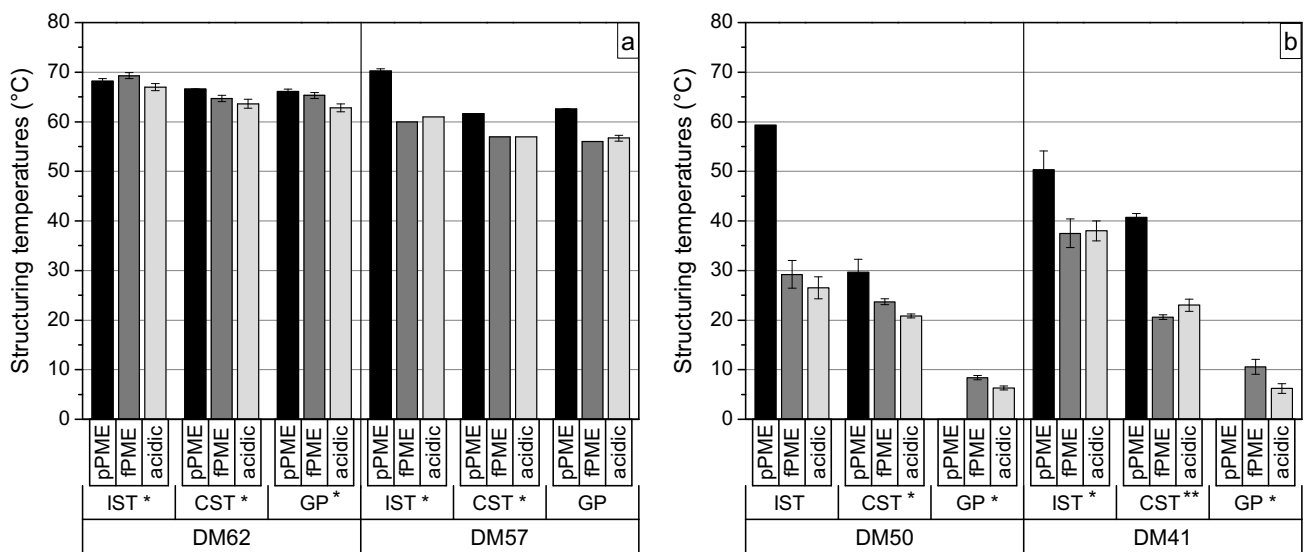


Fig. 3    Influence of pectin demethoxylation method on the structuring temperatures. Sugar-acid gels of DM62 and DM57 samples (HMP) in (a) as well as sugar-calcium gels of DM50 and DM42 samples (LMP) in (b). Structuring temperatures: IST = initial structuring temperature, CST = critical structuring temperature and GP = gel point. Significant differences: * for P < 0.05 and ** for P < 0.01.

### 3.2.2 Gel properties after cooling

The viscoelastic properties of the pectin gels after cooling (determined at 10 °C) were characterized by the $\tan\delta_{end}$. In general, the higher the $\tan\delta_{end}$ value, the more the viscous properties dominate over the elastic gel properties. The $\tan\delta_{end}$ values were similar in the two HMP groups (Fig. 4a). The $\tan\delta_{end}$ value of the DM57 samples was slightly lower than that of the DM62 samples; this indicates that the gel structure of the DM57 samples is more elastic.
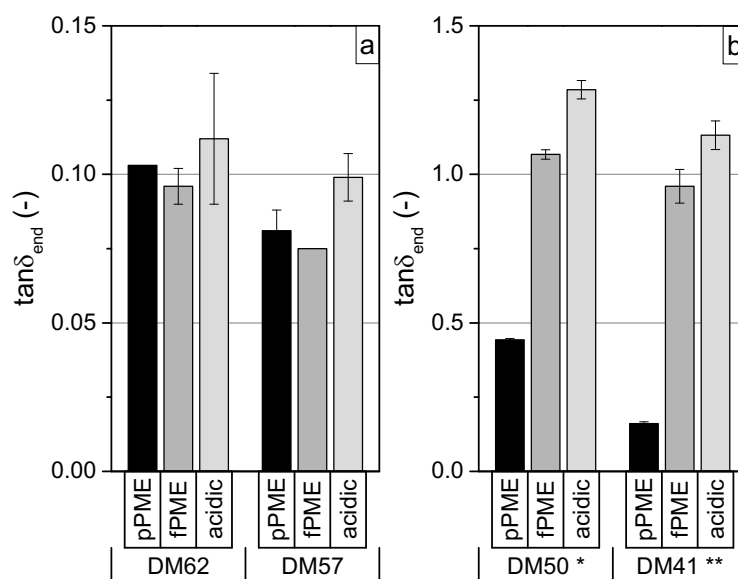
*Fig. 4    Gel properties of pectin gels determined at the end of gelation (10 °C) in dependence on the demethoxylation method. Loss factor (tan$\delta_{end}$) of sugar-acid gels of DM62 and DM57 samples (HMP) in (a) as well as loss factor of sugar-calcium gels of DM50 and DM42 samples (LMP) in (b). Significant differences: * for P < 0.05 and ** for P < 0.01.*

The gels of the LMP samples differed significantly at the end of the cooling process (Fig. 4b), with tan$\delta_{end}$ values of p-pectin << f-pectin < a-pectin at both DM41 and DM50. The DM41 values were slightly lower than those at DM50 (Fig. 4b). For the pPME treated LMP samples, tan$\delta_{end}$ values were below 1, elastic properties dominated, and a gel structure was formed. Comparing the f- and a-pectin samples at 10 °C, the f-pectin had the lower tan$\delta_{end}$ value, however, the tan$\delta_{end}$ was above 1.0 for both, indicating dominant viscous properties. After additional cooling to 5 °C, the tan$\delta_{end}$ decreased below 1.0, but the values for the f-pectin samples (0.826 for F49, 0.805 for F42) were still lower than those of the a-pectin samples (0.914 for A50 and 0.959 for A42).

## 4. Discussion

### 4.1 Comparison of molecular parameters of the demethoxylated pectin samples

The varying intrinsic viscosity and the change in the molecular weight of the modified pectin samples result from different effects. Acidic demethoxylation of pectin was accompanied by a certain depolymerisation. Depolymerisation by backbone hydrolysis as side reaction was found also by Diaz, Anthon, & Barrett (2007) as well as Fraeye, Duvetter, et al. (2010). This effect was limited and increased slowly with decreasing DM, since the demethoxylation was performed at room temperature using moderate concentrations of HCl. The depolymerisation of the p-pectin samples probably was caused by side reactions of depolymerizing enzymes and by ß-elimination. Commercial PME often contain small amounts of depolymerizing enzymes such as polygalacturonase (Benen, Vincken, & van Alebeek, 2002; Christiaens, Van Buggenhout, et al., 2016). A possible side activity in the self-prepared (non-aseptic) PME was confirmed by electrophoresis patterns (not shown). For instance, bands at 65 kDa were detected, outside of the typical range for pPME between 25 and 54 kDa (Benen, Alebeek, Voragen, & Visser, 2002). Polygalacturonases vary in molecular mass between 30 – 75 kDa (Benen & Visser, 2002b) and pectate and pectin lyases between 22.8 – 82.3 kDa (Benen & Visser, 2002a), they might have formed the bands at 65 kDa. In general, depolymerizing enzymes were not found in young developed sweet oranges except of the abscission zone of the fruit (Burns, Lewandowski, Nairn, & Brown, 2002), and should not be contained in the pPME extract. However, a fungal infection of orange fruits, for instance by *Botrytis cinerea* (Lionetti et al., 2017)*,* was possible during transport and storage. *B. cinerea* genome indicates a large array of depolymerizing enzymes such as polygalacturonase or pectin lyase (Blanco-Ulate et al., 2014) and the enzymes might have been co-extracted from the fruits. In case of the p-pectin samples, beside the effect of depolymerizing enzymes also ß-elimination was possible. This reaction is favored at higher temperatures and at a pH above 8, but was detected also at pH 6 (Diaz et al., 2007). Thus, ß-elimination during the pPME-treatment at pH 7.4 and 30 °C was possible. This reaction, as well as enzymatic depolymerisation, increased with reaction time. As a result, the intrinsic viscosity and molecular weight of p-pectin of group DM41 (P40) was the lowest (Table 1) and the MW distribution the broadest (Fig. 1) of all modified pectin samples.

The differences in the sodium content resulted from the modification procedures. For the a-pectin samples, the sodium ions contained in the commercial pectin were widely removed during demethoxylation (Table 1). For the enzymatically demethoxylated pectin samples, the increased sodium content resulted from NaOH used to keep pH constant during the pH-stat method. The number of free carboxyl groups increased during demethoxylation, and some of these groups dissociated and bound sodium ions. Due to the higher pH (7.4 > 4.4), treatment with pPME required

more NaOH than demethoxylation with fPME and resulted in a higher concentration of sodium ions in HMP samples (DM61 and DM57). The higher the start pH, the more NaOH was necessary to adjust the initial pH and to keep it constant during modification. However, the difference in the sodium content of the p-pectin and f-pectin samples vanished with decreasing DM, and the content in the LMP samples became similar. Though more sodium ions were added for preparing samples of p-pectin in comparison to f-pectin during the longer reactions, above a certain threshold the ions were in surplus and were removed during further processing. The residual ions were strongly bound, and their content was independent on the amount added before.

Calcium sensitivity, used in order to characterize the distribution of free carboxyl groups after demethoxylation (Dominiak et al., 2014; Glahn & Rolin, 1996; Limberg et al., 2000a), was high for the p-pectin samples and confirmed the desired block-wise distribution. Calcium sensitivity increased with decreasing DM due to increased block formation of free carboxyl groups with demethoxylation. The calcium sensitivity was low and nearly independent of DM for the HMP after fPME and acidic treatment. The values confirmed a random distribution of the free carboxyl groups for these samples.

*4.2 Comparison of structure formation and gel properties*

The method of gel preparation (sugar-acid gels for the HMP and calcium gels for the LMP) as well as the viscoelastic properties of the resulting gels differed, therefore the two types of gels will be discussed separately.

*4.2.1 Gelation of sugar-acid gels*

Generally, HMP form gels in the presence of sugar and acid (pH < 3.5). These conditions promote the structure formation by reducing the distance between pectin molecules. The low pH decreases the pectin dissociation and the electrostatic repulsion of the negatively charged carboxyl groups. Sodium ions might have a comparable effect, they shield the dissociated carboxyl groups and reduce the electrostatic repulsion (Einhorn-Stoll et al., 2015; Ström & Goh, 2013; Ström, Schuster, & Goh, 2014). Initially, at a temperature > 50 °C, hydrophobic interactions between ester groups dominate. They are reduced during cooling, and are replaced by hydrogen bonds between carboxyl and hydroxyl groups (Oakenfull & Fenwick, 1977). These two interaction types should be independent of the number of free carboxyl group. However, HMP with a partly block-wise distribution of free carboxyl groups (i.e. pectin prepared by pPME) also undergoes ionotropic gelation immediately after the start of cooling (Christiaens, Buggenhout, et al., 2016; Fraeye, Colle, et al., 2010; Löfgren et al., 2005; Ngouémazong, Jolie, et al., 2012), and in these cases the DM becomes important.

In the presented investigation, the difference of the structuring temperature of the samples at DM62 was small (Fig. 2a and 3a). It was attributed to differences in free carboxyl group distribution (block-

wise or random) and/or to the sodium ion content, which was in P61 > F62 > A62. Despite the high calcium sensitivity of p-pectin samples (Table 2), their more block-wise distribution had only a small effect on gelation. The free carboxyl groups in some blocks of the P61 possibly were shielded by sodium ions via counterion condensation (Celus et al., 2017; Irani, Owen, Mercadante, & Williams, 2017; Siew, Williams, & Young, 2005) and, as a result, they were insufficient for additional ionotropic gelation.

In the DM57 group, the structure formation of the p-pectin samples started at significantly higher temperatures than that of the pectin samples with a statistical distribution (Fig. 2a and 3a). The higher number and/or longer blocks of free carboxyl groups supported ionotropic gelation as well as the formation of longer regions of hydrogen bonding (Fraeye, Colle, et al., 2010; Luzio & Cameron, 2008; Ström et al., 2007; Willats et al., 2001). The necessary calcium ions for the supportive ionotropic gelation were contained in the OP86 (1.49 mg/g). The other possible explanation, the formation of longer hydrogen bonding regions in blocks of free carboxyl groups, is not reasonable at 60 – 70 °C when the gelation of p-pectin started.

For the two HMP with more randomly distributed free carboxyl groups (fPME and acidic treatment), the structuring temperatures differed slightly at DM62 and were similar at DM57 (Fig. 3a). However, a difference in the start of gelation (IST), corresponding to a difference in pectin sodium content (12 mg/g for f-pectin vs. 0 mg/g for a-pectin samples), was found at both DM. The sodium ions partly shielded the dissociated carboxyl groups, reduced repulsion between pectin chains and allowed an earlier structure formation for the f-pectin samples. Schmelter, Vreeker and Klaffke (2001) observed a similar effect during gelation. In addition, they found, that inactivated fungal PME had an influence on gelation by accelerating structure formation and strengthening the final gels compared to pectin without PME-protein.

The DM of the pectin samples had an impact on the viscoelastic character of the final gels after cooling to 10 °C (Fig. 4a). They were slightly stronger (more elastic than viscous) at lower DM. This is explained by the higher number of free carboxyl groups at lower DM, resulting in more hydrogen bonds. The influence of blocks of free carboxyl groups was insignificant for sugar-acid gels.

 viscoelastic properties of the DM62 samples differed visibly but not significantly. An order of a-pectin > p-pectin > f-pectin was detected for $\tan\delta_{end}$ and became more pronounced for the DM57 samples, however, the total differences were small. This was possibly an influence of different molecular weights, which were significantly lower for the a-pectin than for the p- and f-pectin (Table 1).

### 4.2.2 Gelation of sugar-calcium gels

The gelation mechanism of sugar-calcium gels differs from that of sugar-acid gels. The formation of hydrophobic interactions above 50 °C is reduced because there are fewer ester groups. Thus,

gelation starts later but is accelerated during cooling by increased hydrogen bond formation below 50 °C. The additional ionotropic gelation via calcium bridges starts immediately with cooling and is stronger in LMP samples than in most HMP because of the higher number of subsequent free carboxyl groups (Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2006; Fraeye, Colle, et al., 2010; Lootens et al., 2003; Ngouémazong, Jolie, et al., 2012).

As expected for the presented work, the structure formation and the gel properties after cooling of the three pectin types differed strongly also for LMP gels in dependence on the pattern of free carboxyl groups. In the DM50 group, the gelling process of the p-pectin samples with a block-wise distribution began at about 60 °C. This was 30 K above the temperature of the according a- and f-pectin samples (Fig. 2b and 3b), since the block-wise distribution allowed additional rapid ionotropic gelation. Despite of the added calcium ions, ionotropic gelation was still limited in the a-n and f-pectin samples at DM50, due to their still mostly randomly distributed free carboxyl groups. The course of the structure formation of the three pectin types also differed (Fig. 2b and 3b): The f- and a-pectin gels showed a steeper increase in gelation velocity below 30 °C, since hydrogen bond formation was dominating in this temperature region.

The structure formation of the DM41 group was delayed in comparison to the DM50 group for the p-pectin but accelerated for the other two samples, and the difference between the three types generally decreased. On the one hand, the rapid formation of hydrophobic interactions was further reduced by the decreasing DM. On the other hand, the total demethoxylation was so high, that also the f-pectin and a-pectin now were able to form longer blocks of free carboxyl groups and to undergo more ionotropic gelation.

Discussing the properties of cooled LMP gels, it has to be considered, that these measurements generally were made at 10 °C. Gel points were found for f- and a-pectin samples, however, only during cooling to 5 °C, and the p-pectin samples showed no gel point at all due to pre-gelation. Nevertheless, the final p-pectin gels at 10 °C were the most elastic of all tested samples (Fig. 4b). The higher length and number of junction zones, formed in blocks of free carboxyl groups of the pPME treated samples by calcium bridges, strongly affected their gel properties. The results agree with those of Fraeye et al. (2009), Löfgren et al. (2005), Ngouémazong, Tengweh, et al. (2012) and Rolin (2002). Comparing the properties of the f- and a-pectin gels, a-pectin samples formed a significantly more viscous and less elastic structure than f-pectin samples. However, since the gel points of these samples were mainly found below 10 °C, gelation was not completed at the final measurement temperature. After cooling to 5 °C (below the GP), the tan$\delta_{end}$ values of the f-pectin gels again were lower than those of the a-pectin samples. The lower gel point temperatures for the a-pectin gels also explained the higher viscosity of these gels. The results confirm an influence of sodium ions and possibly also of inactivated fPME for LMP, as described by Schmelter et al. (2001). The f- and a-pectin samples varied, however, not only in their content of sodium ions and in the

presence of inactivated fPME, they differed additionally in intrinsic viscosity and molecular weight. The influence of these parameters on pectin gelation in general is not sufficiently known and requires further investigation.

*4.3 Final remarks*

The results of the study are summarized as follows: (1) The block-wise or random distribution of free carboxyl groups affected the velocity of structure formation in sugar containing pectin gels. Samples demethoxylated by pPME contained longer blocks of free carboxyl groups, and their gelation started earlier than that of samples demethoxylated by fPME or acid with a more random distribution. The differences increased with decreasing DM, since more free carboxyl groups in the pPME samples formed longer blocks, which supported ionotropic gelation. The final HMP gels had a similar viscoelasticity that was mostly independent of the distribution of the free carboxyl groups and, thus, on the method of demethoxylation. In contrast, the LMP gels of the p-pectin samples were stronger and more elastic than those of the f- and a-pectin samples, thus the demethoxylation method had an impact on the gel structure. (2) The treatments with fPME and acid both resulted in pectin with a random distribution of free carboxyl groups, but the pectin samples showed differences in sodium content and intrinsic viscosity as well as in the presence of inactivated PME. As a result, the structure formation varied as follows: The gelation of f-pectin samples started earlier than that of a-pectin samples, since sodium ions reduced the electrostatic repulsion between pectin molecules in the f-pectin samples and accelerated the formation of intermolecular junction zones. The final gels of f-pectin samples were less viscous and more elastic than those of a-pectin samples.

As a result of the presented work, a general influence of the method of demethoxylation on pectin gelation and gel properties was found. It is based on variations in the pattern of free carboxyl groups and, additionally, on differences of the sodium ion content and the molecular weight.

## 5. Conclusions

The presented work was focused on the effect of type and extent of the demethoxylation of pectin on its gelation process and on the properties of the final gel. Pectin from different modification methods varied in the pattern of the free carboxyl groups as well as in the sodium ion content and the molecular weight, and these differences affected the pectin gelation.

As expected, the pattern of the free carboxyl groups was the dominating factor for the gelation kinetic at any DM. It was, however, less important for the final gel properties, in particular at high DM. Beside this main factor, also the side effects of the demethoxylation reactions, molecular weight reduction, sodium ion content and possibly also the content of inactivated enzymes, had an impact on the gelation kinetic as well as on all final gel properties. The resulting properties were crucial in particular for the different structure formation and gel properties of the two groups of pectin with statistical distribution of free carboxyl groups.

The presented results show in detail the impact of the pectin modification type on the gelation process and the final gel properties in a broad range of DM in sugar-acid as well as sugar-calcium gel systems. They should be considered, when choosing the optimum pectin for a special application.

The impact of molecular weight and sodium ions on pectin gelation at different DM is still not completely understood and requires further investigation. In particular, the independent effects of the single factors are the subject of the ongoing work.

*List of abbreviations*

a-/A    acidic treated sample

CST    critical structuring temperature

dG'/dt  structuring velocity

DM    degree of methoxylation

f-/F    fPME treated sample

fPME  PME of fungal origin

G'      storage modulus

G''    loss modulus

GalA  galacturonic acid

GC    galacturonic acid content

GP    gel point temperature

GPC    gel permeation chromatography

HMP  high-methoxylated pectin

IST    initial structuring temperature

LMP    low-methoxylated pectin

MW    molecular weight

p-/P    pPME treated sample

PME    pectin methylesterase

pPME  PME of plant origin

OP    original pectin

$\tan\delta_{end}$ loss factor at end of measurement

$[\eta]$      intrinsic viscosity

*References*

Arbaisah, S. M., Asbi, B. A., Junainah, A. H., & Jamilah, B. (1997). Purification and properties of pectinesterase from soursop (Anona muricata) pulp. *Food Chemistry*, *59*(1), 33–40. https://doi.org/10.1016/S0308-8146(96)00043-X

Axelos, M. A. V., Garnier, C., Renard, C. M. G. C., Thibault, J.-F., Visser, J., & Voragen, A. G. J. (1996). Interactions of pectins with multivalent cations: Phase diagrams and structural aspects. In W. Y. Aalbersberg, R. J. Hamer, P. Jasperse, H. H. J. de Jongh, C. G. de Kruifand, P. Walstra, & F. A. de Wolf (Eds.), *Progress in Biotechnology* (Vol. Volume 14, pp. 35–45). Elsevier.

Bäuerle, G., Otterbach, G., Grieschner, K., & Baumann, G. (1977). Bestimmungen des Polyuronidgehaltes und des Veresterungsgrades des Pektinanteiles in Handelspektinpräparaten, Apfelsäften und Apfelmaceraten. *Deutsche Lebensmittelrundschau*, *73*(9), 281–286.

Benen, J. A. E., Vincken, J.-P., & van Alebeek, G.-J. (2002). Microbial pectinases. In G. B. Seymour & J. P. Knox (Eds.), *Pectins and Their Manipulation* (1 edition, pp. 170–221). Oxford: Blackwell Publishing.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*(2), 484–489. https://doi.org/10.1016/0003-2697(73)90377-1

Burey, P., Bhandari, B. R., Howes, T., & Gidley, M. J. (2008). Hydrocolloid Gel Particles: Formation, Characterization, and Application. *Critical Reviews in Food Science and Nutrition*, *48*(5), 361–377. https://doi.org/10.1080/10408390701347801

Capel, F., Nicolai, T., Durand, D., Boulenguer, P., & Langendorff, V. (2006). Calcium and acid induced gelation of (amidated) low methoxyl pectin. *Food Hydrocolloids*, *20*(6), 901–907. https://doi.org/10.1016/j.foodhyd.2005.09.004

Cardoso, S. M., Coimbra, M. A., & Lopes da Silva, J. A. (2003). Temperature dependence of the formation and melting of pectin–Ca2+ networks: a rheological study. *Food Hydrocolloids*, *17*(6), 801–807. https://doi.org/10.1016/S0268-005X(03)00101-2

Christiaens, S., Van Buggenhout, S., Houben, K., Jamsazzadeh Kermani, Z., Moelants, K. R. N., Ngouémazong, E. D., … Hendrickx, M. E. G. (2016). Process-Structure-Function Relations of Pectin in Food. *Critical Reviews in Food Science and Nutrition*, *56*(6), 1021–1042. https://doi.org/10.1080/10408398.2012.753029

da Silva, J. A. L., Gonçalves, M. P., & Rao, M. A. (1995). Kinetics and thermal behaviour of the structure formation process in HMP/sucrose gelation. *International Journal of Biological Macromolecules*, *17*(1), 25–32. https://doi.org/10.1016/0141-8130(95)93514-X

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*(1–4), 135–145. https://doi.org/10.1016/S0008-6215(99)00093-2

Diaz, J. V., Anthon, G. E., & Barrett, D. M. (2007). Nonenzymatic Degradation of Citrus Pectin and Pectate during Prolonged Heating:  Effects of pH, Temperature, and Degree of Methyl

Esterification. *Journal of Agricultural and Food Chemistry*, *55*(13), 5131–5136. https://doi.org/10.1021/jf0701483

Dominiak, M., Søndergaard, K. M., Wichmann, J., Vidal-Melgosa, S., Willats, W. G. T., Meyer, A. S., & Mikkelsen, J. D. (2014). Application of enzymes for efficient extraction, modification, and development of functional properties of lime pectin. *Food Hydrocolloids*, *40*, 273–282. https://doi.org/10.1016/j.foodhyd.2014.03.009

Einhorn-Stoll, U., Glasenapp, N., & Kunzek, H. (1996). Modified pectins in whey protein emulsions. *Food / Nahrung*, *40*(2), 60–67. https://doi.org/10.1002/food.19960400203

Einhorn-Stoll, U., Kastner, H., Hecht, T., Zimathies, A., & Drusch, S. (2015). Modification and physico-chemical properties of citrus pectin – Influence of enzymatic and acidic demethoxylation. *Food Hydrocolloids*, *51*, 338–345. https://doi.org/10.1016/j.foodhyd.2015.05.031

Einhorn-Stoll, U., Kastner, H., & Senge, B. (2012). Comparison of Molecular Parameters; Material Properties and Gelling behaviour of Commercial Citrus Pectins. In P. A. Williams & G. O. Phillips (Eds.), *Gums and Stabilisers for the Food Industry 16* (pp. 199–206). Retrieved from https://doi.org/10.1039/9781849734554-00199

Einhorn-Stoll, U., Kastner, H., Urbisch, A., Kroh, L. W., & Drusch, S. (2018). Thermal degradation of citrus pectin in low-moisture environment - Influence of acidic and alkaline pre-treatment. *Food Hydrocolloids*. https://doi.org/10.1016/j.foodhyd.2018.02.030

Evageliou, V., Richardson, R. K., & Morris, E. R. (2000). Effect of pH, sugar type and thermal annealing on high-methoxy pectin gels. *Carbohydrate Polymers*, *42*(3), 245–259. https://doi.org/10.1016/S0144-8617(99)00191-5

Fraeye, I., Doungla, E., Duvetter, T., Moldenaers, P., Van Loey, A., & Hendrickx, M. (2009). Influence of intrinsic and extrinsic factors on rheology of pectin–calcium gels. *Food Hydrocolloids*, *23*(8), 2069–2077. https://doi.org/10.1016/j.foodhyd.2009.03.022

Fraeye, I., Duvetter, T., Doungla, E., Van Loey, A., & Hendrickx, M. (2010). Fine-tuning the properties of pectin–calcium gels by control of pectin fine structure, gel composition and environmental conditions. *Trends in Food Science & Technology*, *21*(5), 219–228. https://doi.org/10.1016/j.tifs.2010.02.001

Garnier, C., Axelos, M. A. V., & Thibault, J.-F. (1991). Dynamic viscoelasticity and thermal behaviour of pectin—calcium gels. *Food Hydrocolloids*, *5*(1), 105–108. https://doi.org/10.1016/S0268-005X(09)80293-2

Glahn, P.-E., & Rolin, C. (1996). Properties and food uses of pectin fractions. In G. O. Phillips, P. A. Williams, & D. J. Wedlock (Eds.), *Gums and Stabilisers for the Food Industry 8* (pp. 393–402). Oxford: Oxford University Press.

Holst, P. S., Kjøniksen, A.-L., Bu, H., Sande, S. A., & Nyström, B. (2006). Rheological properties of pH-induced association and gelation of pectin. *Polymer Bulletin*, *56*(2–3), 239–246. https://doi.org/10.1007/s00289-005-0489-8

Jensen, S., Rolin, C., & Ipsen, R. (2010). Stabilisation of acidified skimmed milk with HM pectin. *Food Hydrocolloids*, *24*(4), 291–299. https://doi.org/10.1016/j.foodhyd.2009.10.004

Kastner, H., Einhorn-Stoll, U., & Drusch, S. (2017). Structure formation in sugar containing pectin

gels - Influence of gel composition and cooling rate on the gelation of non-amidated and amidated low-methoxylated pectin. *Food Hydrocolloids*, *73*(Supplement C), 13–20. https://doi.org/10.1016/j.foodhyd.2017.06.023

Kastner, H., Einhorn-Stoll, U., & Senge, B. (2012a). New parameters for the examination of the pectin gelation process. In P. A. Williams & G. O. Phillips (Eds.), *Gums and Stabilisers for the Food Industry 16* (pp. 191–197). Retrieved from https://doi.org/10.1039/9781849734554-00191

Kastner, H., Einhorn-Stoll, U., & Senge, B. (2012b). Structure formation in sugar containing pectin gels – Influence of Ca2+ on the gelation of low-methoxylated pectin at acidic pH. *Food Hydrocolloids*, *27*(1), 42–49. https://doi.org/10.1016/j.foodhyd.2011.09.001

Kastner, H., Kern, K., Wilde, R., Berthold, A., Einhorn-Stoll, U., & Drusch, S. (2014). Structure formation in sugar containing pectin gels – Influence of tartaric acid content (pH) and cooling rate on the gelation of high-methoxylated pectin. *Food Chemistry*, *144*, 44–49. https://doi.org/10.1016/j.foodchem.2013.06.127

Kim, Y., Teng, Q., & Wicker, L. (2005). Action pattern of Valencia orange PME de-esterification of high methoxyl pectin and characterization of modified pectins. *Carbohydrate Research*, *340*(17), 2620–2629. https://doi.org/10.1016/j.carres.2005.09.013

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000a). Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from A. Niger. *Carbohydrate Research*, *327*(3), 293–307. https://doi.org/10.1016/S0008-6215(00)00067-7

Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000b). 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*(3), 321–332. https://doi.org/10.1016/S0008-6215(00)00068-9

Löfgren, C., Guillotin, S., Evenbratt, H., Schols, H., & Hermansson, A.-M. (2005). Effects of calcium, pH, and blockiness on kinetic rheological behavior and microstructure of HM pectin gels. *Biomacromolecules*, *6*(2), 646–652. https://doi.org/10.1021/bm049619+

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*(3), 237–244. https://doi.org/10.1016/S0268-005X(02)00056-5

Lopes da Silva, J. A., & Rao, M. A. (2007). Rheological Behavior of Food Gels. In *Rheology of Fluid and Semisolid Foods* (pp. 339–401). Springer US. https://doi.org/10.1007/978-0-387-70930-7_6

Luzio, G. A., & Cameron, R. G. (2008). Demethylation of a model homogalacturonan with the salt-independent pectin methylesterase from citrus: Part II. Structure–function analysis. *Carbohydrate Polymers*, *71*(2), 300–309. https://doi.org/10.1016/j.carbpol.2007.05.038

May, C. D. (1990). Industrial pectins: Sources, production and applications. *Carbohydrate Polymers*, *12*(1), 79–99. https://doi.org/10.1016/0144-8617(90)90105-2

Mende, S., Peter, M., Bartels, K., Dong, T., Rohm, H., & Jaros, D. (2013). Concentration

dependent effects of dextran on the physical properties of acid milk gels. *Carbohydrate Polymers*, *98*(2), 1389–1396. https://doi.org/10.1016/j.carbpol.2013.07.072

Ngouémazong, D. E., Jolie, R. P., Cardinaels, R., Fraeye, I., Van Loey, A., Moldenaers, P., & Hendrickx, M. (2012). Stiffness of Ca2+-pectin gels: combined effects of degree and pattern of methylesterification for various Ca2+ concentrations. *Carbohydrate Research*, *348*(Supplement C), 69–76. https://doi.org/10.1016/j.carres.2011.11.011

Ngouémazong, D. E., Kabuye, G., Fraeye, I., Cardinaels, R., Van Loey, A., Moldenaers, P., & Hendrickx, M. (2012). Effect of debranching on the rheological properties of Ca2+–pectin gels. *Food Hydrocolloids*, *26*(1), 44–53. https://doi.org/10.1016/j.foodhyd.2011.04.009

Ngouémazong, D. E., Tengweh, F. F., Fraeye, I., Duvetter, T., Cardinaels, R., Van Loey, A., … Hendrickx, M. (2012). Effect of de-methylesterification on network development and nature of Ca2+-pectin gels: Towards understanding structure–function relations of pectin. *Food Hydrocolloids*, *26*(1), 89–98. https://doi.org/10.1016/j.foodhyd.2011.04.002

Oakenfull, D., & Fenwick, D. E. (1977). Thermodynamics and mechanism of hydrophobic interaction. *Australian Journal of Chemistry*, *30*(4), 741–752. https://doi.org/10.1071/CH9770741

Oakenfull, D., & Scott, A. (1984). Hydrophobic interaction in the gelation of high methoxyl pectins. *Journal of Food Science*, *49*(4), 1093–1098. https://doi.org/10.1111/j.1365-2621.1984.tb10401.x

Ralet, M.-C., Crépeau, M.-J., 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*, *16*(2), 191–201. https://doi.org/10.1016/S1369-703X(03)00037-8

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, *57*(6), 929–967. https://doi.org/10.1016/S0031-9422(01)00113-3

Rolin, C. (2002). Commercial pectin preparartion. In G. B. Seymour & J. P. Knox (Eds.), *Pectins and Their Manipulation* (1 edition, pp. 222–241). Oxford: Blackwell Publishing.

Rolin, C., & Vries, J. D. (1990). Pectin. In P. Harris (Ed.), *Food Gels* (pp. 401–434). Springer Netherlands. https://doi.org/10.1007/978-94-009-0755-3_10

Ström, A., & Goh, S. M. (2013). Rheological Characterisation of Acid Pectin Samples in Absence and Presence of Monovalent Ions. In *Annual transactions - The Nordic Rheology Society* (Vol. 21, pp. 223–230). Copenhagen. Retrieved from https://nordicrheologysociety.org/Content/Transactions/2013/32_StromRheologicalCharacterisationofAcidPectinSamplesinAbsenceandPresence.pdf

Ström, A., Ribelles, P., Lundin, L., Norton, I., Morris, E. R., & Williams, M. A. K. (2007). Influence of pectin fine structure on the mechanical properties of calcium−pectin and acid−pectin gels. *Biomacromolecules*, *8*(9), 2668–2674. https://doi.org/10.1021/bm070192r

Ström, A., Schuster, E., & Goh, S. M. (2014). Rheological characterization of acid pectin samples in the absence and presence of monovalent ions. *Carbohydrate Polymers*, *113*, 336–343. https://doi.org/10.1016/j.carbpol.2014.06.090

Thakur, B. R., Singh, R. K., Handa, A. K., & Rao, D. M. A. (1997). Chemistry and uses of pectin —
A review. *Critical Reviews in Food Science and Nutrition*, *37*(1), 47–73.
https://doi.org/10.1080/10408399709527767

Thibault, J.-F., & Ralet, M.-C. (2003). Physico-chemical properties of pectins in the cell walls and
after extraction. In F. Voragen, H. Schols, & R. Visser (Eds.), *Advances in Pectin and
Pectinase Research* (pp. 91–105). Springer Netherlands. https://doi.org/10.1007/978-94-
017-0331-4_7

Tsoga, A., Richardson, R. K., & Morris, E. R. (2004). Role of cosolutes in gelation of high-methoxy
pectin. Part 1. Comparison of sugars and polyols. *Food Hydrocolloids*, *18*(6), 907–919.
https://doi.org/10.1016/j.foodhyd.2004.03.001

Vetter, S., & Kunzek, H. (2003). The influence of the sequential extractions on the structure and
the properties of single cell materials from apples. *European Food Research and
Technology*, *217*(5), 392–400. https://doi.org/10.1007/s00217-003-0767-3

Vincent, R. R., & Williams, M. A. K. (2009). Microrheological investigations give insights into the
microstructure and functionality of pectin gels. *Carbohydrate Research*, *344*(14), 1863–
1871. https://doi.org/10.1016/j.carres.2008.11.021

Voragen, A. G. J., Coenen, G.-J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile
polysaccharide present in plant cell walls. *Structural Chemistry*, *20*(2), 263.
https://doi.org/10.1007/s11224-009-9442-z

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

Willats, W. G. T., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: cell biology and
prospects for functional analysis. In *Plant Cell Walls* (pp. 9–27). Springer, Dordrecht.
https://doi.org/10.1007/978-94-010-0668-2_2

Willats, W. G. T., Orfila, C., Limberg, G., Buchholt, H. C., Alebeek, G.-J. W. M. van, Voragen, A. G.
J., … 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. *Journal of
Biological Chemistry*, *276*(22), 19404–19413. https://doi.org/10.1074/jbc.M011242200

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*(7), 1777–1781. https://doi.org/10.1021/jf0259112

Yapo, B. M., & Gnakri, D. (2015). Pectic polysaccharides and their functional properties. In K. G.
Ramawat & J.-M. Mérillon (Eds.), *Polysaccharides - Bioactivity and Biotechnology* (pp.
1729–1746). Springer International Publishing. https://doi.org/10.1007/978-3-319-03751-
6_62-1