**Evaluation of cation-facilitated pectin-gel properties: Cryo-SEM visualisation
and rheological properties**

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Abstract

To provide more insight into the cation facilitated pectin-gel structure-function relationship, the microstructure of pectin-gels was explored using cryo-scanning electron microscopy (Cryo-SEM) in combination with physiochemical and rheological analysis. Pectin with different degrees of methylesterification, DM, was obtained from commercial citrus pectin (CP) and sugar beet pectin (SBP) by action of carrot pectin methylesterase. Pectin characterisation confirmed that pectin properties other than DM were unaltered and pectin from the two sources exhibited different structures. Cryo-SEM visualisation of pectin-gels prepared with different cations revealed that the network density increased with decreasing DM and increasing pH, pectin and cation-concentration. At a given DM, the more linear and less acetylated pectin (CP) exhibited a more intertwined fibrous network compared to the highly branched and more acetylated pectin (SBP). Furthermore, gel-microstructure was influenced by type of cation with Zn^{2+} , Fe^{2+} and Ca^{2+} -pectin gels showing a more intertwined network compared to Mg^{2+} -pectin gels. Finally, rheological properties of gels complementing Cryo-SEM observations showed that as the network density increased, the gel-stiffness (G') increased.

Keywords: Citrus pectin ; Sugar beet pectin; Degree of methylesterification; Ca^{2+} , Zn^{2+} , Fe^{2+} and Mg^{2+} -mediated gels; Gel-stiffness.

1. Introduction

Gel-based foods constitute an important part of the food industry with products such as jams and jellies being largely consumed. These products are convenient, easy to transport and usually exhibit a unique structure/consistency which has been linked to the presence of pectin, a natural polymer found in the cell walls of fruits. Next to the food industry, gelling is employed in other industries including the cosmetics industry (e.g. in hair gels and bathing gels), and in the pharmaceutical industry. According to Oakenfull et al. (1997), gels are usually defined as a three-dimensional network in which a fluid medium is entrapped. Therefore, to form a gel, at a molecular level, three elements are important; junction zones where polymer molecules are joined together, inter-junction segments of polymers that are relatively mobile, and water entrapped in the polymer network (Thakur, Singh, & Handa, 1997). In case of low acid foods, Ca^{2+} -ions are commonly used as cross-linking agent when commercially extracted pectin, either by itself or in combination with other biopolymers is usually employed as a gelling agent (Lopes da Silva & Rao, 2006).

Pectin is a heteropolysaccharide present in the cell wall of fruits and vegetables where it is predominantly made up of galacturonic acid (GalA) residues, with varying proportions of GalA residues present as methyl esters, while a specific amount of neutral sugars might be present as side chains (Voragen, Coenen, Verhoef, & Schols, 2009). This complex polymer is made up of three major substructures, homogalacturonan (HG), rhamnogalacturonan II (RG-II) and rhamnogalacturonan I (RG-I). The GalA residues within the backbone of the HG pectic polymer (the major pectic substructure) may be methylesterified at C-6 and/or O-acetylated at O-2 and/or O-3 (Voragen et al., 2009; Sila et al., 2009). In gelling applications, the degree of methylesterification (DM) is an important pectin property and forms the basis to classify pectin

gels as high and low methylesterified pectin gels. Specifically, the non-methylesterified GalA residues can be ionised (negatively charged) depending on the pH of the system giving pectin its polyanionic/ion-binding nature. Consequently, in presence of cations, pectin can be cross-linked intra- and/or inter-molecularly leading to formation of a weak to strong network. Generally, as the DM decreases, the number of non-methylesterified GalA residues increases thereby promoting the ion binding/gelling capacity of pectin. To this extent, Lopes da Silva and Rao. (2006), reports that low methylesterified pectin readily gels (on addition of Ca^{2+} -ions) over a wide range of pH while high methylesterified pectin will only gel at a low pH (<3.5) in presence of a co-solute. The ion binding capacity of pectin is also explored *in situ* to preserve texture of fruits and vegetables. Particularly, in intact plant tissues, endogenous or infused Ca^{2+} -ions cross-link the pectin making the tissues stronger thereby preserving texture upon subsequent processing (Fraeye et al., 2009).

Next to the non-methylesterified carboxylic groups, it is believed that other functional groups of pectin including free hydroxyl groups as well as associated-proteins can contribute to the gelling phenomenon. According to Assifaoui (2015), Zn^{2+} -ions possess a high affinity for the hydroxyl groups and changes in this pectin property can affect its binding capacity. This pectin property is also influenced by several other intrinsic factors including, the pattern of methylesterification (the distribution of methylesterified groups along the HG backbone), neutral sugars and molar mass (Yapo, Lerouge, Thibault, & Ralet, 2007; Kim & Wicker, 2011). Gelation may be greatly influenced by the pattern of methylesterification and it has been reported that at least 10 consecutive blocks of non-methylesterified carboxyl groups are needed for gel formation (Thakur et al., 1997; Voragen et al., 2009; McFeeters, 1985; Kim & Wicker, 2011). Furthermore, neutral sugars, acetyl groups and a high degree of pectin branching are reported to reduce the

cation-binding capacity of pectin (Lopes da Silva & Rao, 2006). In general, pectin structure plays a vital role in gelling applications, and modification of its structure, particularly alterations in the HG may greatly influence the functionality of this polymer. Next to intrinsic factors, several extrinsic factors such as temperature, salt concentration and pH influence the gelling capacity of pectin. For instance, increasing the pH of pectin-based/pectin-containing systems to a pH above the pKa value of pectin (3.38-4.10) (Moore, 1985; Manrique & Lajolo, 2002; Sriamornsak, 2003) allows ionisation of more than 50% of chargeable groups thereby promoting gelation in presence of cations.

Although pectin has been widely explored in gelling applications, most of the related research focuses on the physiochemical structure-function relationship and only limited information is available on the microstructure of the gels. This research therefore explores the microstructure of cation-pectin gels in relation to their physiochemical structure and rheological behaviour in order to provide a broader insight into the structure-function relationship. First, commercial citrus (DM ~ 95%, from Sigma-Aldrich, Belgium) and sugar beet pectin (DM ~ 60%, from CP Kelco, Germany) was demethylesterified to different levels by action of carrot pectin methylesterase (PME) followed by a physiochemical characterisation of the resulting samples. Thereafter, pectin solutions were gelled with Ca^{2+} , Zn^{2+} , Fe^{2+} and Mg^{2+} -ions, visualised under a cryo-scanning electron microscopy (Cryo-SEM) and analysed for their rheological behaviour. Finally, the gel microstructure-rheology-physiochemical structure relationship was established.

2. Materials and methods

2.1 Demethylesterification of pectin

Exactly 0.8% w/v citrus and sugar beet pectin solutions (DM ~ 95% and DM ~ 60%, respectively) were demethylesterified for different time periods using pectin methylesterase

(PME) extracted from carrots (*Daucus carota* cultivar Nantes) as described by Jolie et al. (2009). Thereafter, pH was adjusted to 6 with NaOH (0.1 N or 0.01 N) and the solutions were dialysed (dialysis membranes, molecular weight cut off, MWCO 12-14 kDa) against demineralised water. Subsequently, the samples were lyophilised and stored in a desiccator above P₂O₅ until further use. Purity of lyophilised samples was > 85% (citrus pectin) and > 80% (sugar beet pectin).

2.2 Characterisation of the pectin samples

The samples obtained in Section 2.1 were characterised for different pectin structural properties including the DM, degree of acetylation, pectin content, molar mass, pectin-related sugar content (GalA and neutral sugar content), degree of feruloylation as well as the endogenous mineral content.

First, the degree of methylesterification of pectin was determined using Fourier transform infrared spectroscopy (FT-IR) (Shimadzu FTIR-8400S, Japan) as described by Kyomugasho et al. (2015). Secondly, high-performance size-exclusion chromatography equipped with multi-angle laser light scattering (PN3621, Postnova analytics, Germany) and refractive index (RI) detection (Shodex RI-101, Showa Denko K.K., Kawazaki, Japan) was employed to determine the molar mass (MM) as described by Shpigelman et al. (2014). Next, the content of pectin-related sugars was established. On the one hand, the galacturonic acid (GalA) content was determined as described by Ahmed and Labavitch (1977). Pectin was hydrolysed (in duplicate) with concentrated sulfuric followed by a spectrophotometric analysis of the GalA content as described by Blumenkrantz and Asboe-Hansen (1973). On the other hand, the samples were hydrolysed with trifluoroacetic acid and analysed for the content of pectin-related neutral sugars using high-performance anion-exchange chromatography combined with pulsed amperometric detection as described by Houben et al. (2011). Furthermore, the degree of acetylation and

degree of feruloylation of pectin were determined spectrophotometrically as the ratio of the molar amount of acetyl groups to the molar amount of galacturonic acid content and the ratio of the molar amounts of ferulic acid content to the sum of the molar amounts of arabinose and galactose, respectively. Determination of the degree of acetylation (DAc) was based on a standard procedure provided by the manufacturer of the Megazyme kit used (K-ACETRL, Megazyme) while the degree of feruloylation was determined as described by Georgiev et al. (2012).

Lastly, the amount of protein was determined by estimation of the nitrogen content as described by Hames et al. (2008) while the initial mineral content was analysed by inductively coupled plasma mass spectrometry as detailed by Kyomugasho et al. (2015). The pectin samples were incinerated in a muffle furnace, dissolved in ultrapure water and analysed for concentration of Ca^{2+} , Zn^{2+} , Fe^{2+} and Mg^{2+} -ions.

2.3 Preparation of cation-mediated pectin gels

Exactly, 4 mL of 1% or 2% (w/v) pectin solution was adjusted to either pH 3.5 or 6.2 with NaOH (0.01 N) and used in gel preparation. The pectin solutions were preheated at 40 °C and predetermined concentrations of Ca^{2+} , Mg^{2+} , Zn^{2+} and Fe^{2+} -ions (in solutions) were added dropwise under constant stirring. The resulting gels were cooled to 20 °C and kept at this temperature for at least 6 h. Thereafter, the gels were visualised under Cryo-SEM and analysed for rheological behaviour. Different cation concentrations were added such that specific stoichiometric ratios ($R=2[\text{Ca}^{2+}]/[\text{COO-}]$) (Garnier, Axelos, & Thibault, 1993) of $R = 0$ (no added cations) to $R = 5$ were obtained for each pectin sample. Furthermore, R values between 5 and 20 were also included. Taking into account that R value is influenced by $[\text{COO-}]$, different amounts of cations were added depending on the DM, pectin source as well as pectin

concentration. The Ca^{2+} -ion in the stoichiometric ratio was replaced with Zn^{2+} , Fe^{2+} , and Mg^{2+} -ions where appropriate. In addition to gels prepared based on R values as mentioned above, another set of gels prepared by addition of similar amounts of cations to the samples was analysed.

2.4 Determination of rheological properties

The flow behaviour of the initial pectin solutions and the stiffness of cation-pectin-gels were measured using a stress-controlled rheometer (Physica MCR 501, Anton Paar, Graz, Austria) with Rheoplus software. The cone and plate method (geometry CP 25-2) was used and the measuring gap setting was kept at 0.5 mm. A sufficient amount of gel was carefully loaded (to avoid air bubbles) at the center of the rheometer lower plate and the upper geometry was lowered to the set measuring gap. A solvent trap was then mounted to prevent evaporation (considering the short duration of each test, evaporation was negligible) and oscillatory measurements were then performed (in duplicate) at 20 °C. First, a strain sweep at an angular frequency of 10 rad/s was performed for each sample in order to establish the linear viscoelastic region. Then, a frequency sweep test was performed over a range of 10–0.1 rad/s at a strain within the linear region to determine the storage modulus (G') and the loss modulus (G''). The G' and G'' were then plotted against angular frequency to ascertain the behaviour of the gels. The steady shear viscosity was measured at shear rates of 0.01–10 s^{-1} (for gels) and 1–100 s^{-1} (for pectin solutions).

2.5 Visualisation of the microstructure of cation-mediated pectin gels (Cryo-SEM)

Cryo-scanning electron microscopy (Cryo-SEM) allows for observation up to the level of pore scale of liquid containing samples in a frozen state. In the Cryo-SEM technique, the sample is vitrified with liquid nitrogen and maintained at very low temperature. This preserves the structure of the sample and allows it to remain stable during the microscopy procedure

(Sriamornsak, Thirawong, Cheewatanakornkool, Burapapadh, & Sae-Ngow, 2008). First, a small amount of sample was placed into a slot on a stub with rivets and cryo-vitrified with liquid nitrogen. Thereafter, the vitrified sample was transferred onto a cryo-stage (at -140 °C) in the preparation chamber (PP3010T Cryo-SEM Preparation System, Quorum Technologies, UK), where it was fractured to allow visualisation of the topography/gel-structure. Subsequently, free water was sublimated from the sample at -90 °C under controlled vacuum conditions. After 15-30 min the sample was sputtered with platinum using argon gas to prevent charging during electron beam targeting. Lastly, the sample was transferred on to the SEM stage (JEOL JSM 7100F SEM, JEOL Ltd, Tokyo, Japan) at -140 °C and visualised for distinct topography (Patel et al., 2015).

2.6 Data analysis

Data obtained in this study was statistically analysed using One way ANOVA (analysis of variance) and post hoc Tukey test (SAS Enterprise Guise 5.1, USA). Differences were established at a 95% confidence interval.

3. Results and discussion

3.1 Pectin characterisation

Demethylesterification of citrus pectin (CP) for 30 h, 35 min and 4 min yielded pectin with DMs of 16%, 35% and 65%, respectively while sugar beet pectin (SBP) incubated for 24 h exhibited a DM of 35%. The mother citrus and sugar beet pectin samples exhibited DMs of 95% and 60%, respectively. The decrease in DM with increasing incubation time was due to PME activity. The longer time needed to achieve a DM of 35% in SBP compared to CP can be related to the presence of acetyl groups which hinder the activity of PME (Oosterveld, Beldman, Searle-van Leeuwen, & Voragen, 2000). Further characterisation of these samples revealed that all other

pectin properties were unchanged. In fact, for a given pectin source, no significant changes ($P>0.05$) in pectin properties such as degree of acetylation, molar mass or pectin-related sugar content were observed. However, CP and SBP showed significant differences ($P<0.05$) in their structure. In terms of pectin-related neutral sugars, CP samples exhibited higher amounts of GalA but lower amounts of rhamnose, arabinose, galactose as shown in Table 1. This suggests that CP is predominantly comprised of linear homogalacturonan blocks while SBP is more branched in nature. Additionally, SBP exhibited a significantly higher degree of acetylation and molar mass ($P<0.05$) compared to CP. Lastly, SBP showed only slightly higher protein and degree of feruloylation than CP (Table 1).

After characterisation, the samples were denoted as CP 16, CP 35, CP 65 and CP 95 or SBP 35 and SBP 60 depending on the pectin source and the associated DM. In the subsequent sections, these denotations will be used to refer to the samples.

3.2 Microstructure and flow behaviour of pectin solutions/cation-enriched pectin-gels

Cryo-scanning electron microscopy (Cryo-SEM) was used to visualise the micro/nano-structure of Ca^{2+} , Fe^{2+} , Zn^{2+} and Mg^{2+} -mediated pectin-gels and the micro/nanographs obtained are presented in this section. Considering the low initial amounts of minerals per g of pectin (Ca^{2+} ; 0.01-0.02 mM, Mg^{2+} ; 0.003-0.01 mM, Fe^{2+} ; 5×10^{-4} - 3×10^{-3} mM and Zn^{2+} ; 3×10^{-4} - 3.4×10^{-4} mM), the pectin solutions were gelled by enrichment with these selected minerals. Furthermore, description of the images obtained is based on studies defining pectin and other gels as three-dimensional gels with an intertwined fibrous network (Fishman, Chau, Cooke, & Hotchkiss, 2008; Lopes da Silva & Rao, 2006).

3.2.1 Microstructure of pectin solutions

228 Figure 1A shows the Cryo-SEM images of 1% pectin solutions without addition of cations. In
229 this figure, pectin molecules are mostly associated with amorphous water as indicated by the
230 white regions and the dark spaces are positions previously occupied by the sublimated water.
231 Smaller and intact microstructures were observed for CP 16 and CP 35 but larger and more open
232 microstructures were seen as the DM increased to 65% and 95%. The microstructures of low DM
233 samples are probably related to weak cross-linking in these samples. According to Fraeye et al.
234 (2009), weak networks can be initiated in low DM samples even in presence of low cation
235 concentration considering that low DM samples are highly sensitive to calcium/cations. As the
236 DM increased, sensitivity to the low inherent cation concentrations (cf. Section 3.2) decreased
237 probably leading to much weaker cross-linking (if any) hence the weak and more open
238 structures. In the case of SBP, larger and more open microstructures are evident with strand-like
239 structures which decreased as the DM increased from 35% to 60%. This observation on SBP is
240 probably related to its differences in structure compared to CP. Rheological data complemented
241 the cryo-SEM images. Generally, viscosity decreased with increasing DM (Table 2) in
242 correlation with gel microstructure decreasing as the DM increased (Figure 1A). Additionally,
243 for a given DM, a slightly higher viscosity ($P < 0.05$) was observed for CP than SBP at a shear
244 rate of 1 s^{-1} (Table 2). As the shear rate increased, CP solutions exhibited a pseudo-plastic
245 behaviour (shear-thinning) as shown in Figure 1B and according to Rolin et al. (1998)
246 concentrated pectin solutions (above 0.5%) usually show this behaviour. This observation
247 suggests presence of “a weak network” in CP solutions which is destroyed as the shear rate
248 increases leading to a decrease in viscosity. On the other hand, CP 95 and SBP displayed a
249 Newtonian behaviour, indicating that shear rate has no significant influence on the viscosity
250 thereby suggesting limited cross-linking (if any). It can therefore be concluded that in absence of

added cations, pectin solutions are “structured liquids” whose microstructure and viscosity decrease with increasing DM and are influenced by pectin source.

3.2.2 Microstructure and rheology of Ca²⁺-mediated pectin-gels

Results of Ca²⁺-enriched pectin gels are presented in this section with representative images of selected R values shown in Figure 2A. According to Fraeye et al. (2009), R values of 1-5.7 are required for gelation of LM pectin while other researcher propose an R value of 1 for maximum Ca²⁺-binding. Therefore, several R values were explored and images at R values of 0.5, 1 and 5 are presented here. The lower the R value, the less calcium added. In general, addition of cations promoted formation of strand-like structures/an intertwined fibrous network compared to the case without added cations (Figure 2A compared to Figure 1A). It can be seen from Figure 2A that at a given R value (for example R 0.5), as the DM increased, network formation (indicated by the strand-like structures) decreased. This is related to the fact that as the DM decreases, the number of contiguous non-methylesterified galacturonic acid residues long enough for formation of “egg-boxes” increases and so does calcium binding affinity (Ralet, Crepeau, Buchholt, & Thibault, 2003). In fact, Rolin et al. (1998) reports that the lower the DM, the more firmly metal ions are bound to pectin. For SBP gels, limited cross-linking can be observed even in the case of the low DM sample (SBP 35). These findings were supported by rheology results in Figure 2B showing the evolution of gel stiffness with increasing R value (Ca²⁺-ion concentration). It can be seen that at any given point, CP 16 exhibits consistently higher G' values (p<0.05) than CP 35 and CP 65 indicating an increase in gel stiffness with decreasing DM. Furthermore, for a given DM, CP gels were significantly stiffer (exhibited higher G' values) than SBP gels (p<0.05) as shown for CP 35 and SBP 35 in Figure 2B.

273 When the amount of added Ca^{2+} -ions was increased (R value increased to 1 and 5 as shown in
274 Figure 2A), a more intertwined fibrous network was observed with CP 16 and CP 35 showing
275 the greatest changes. By increasing the Ca^{2+} -ion concentration, cross-linking in the low DM
276 samples is further promoted considering the high number of free carboxyl groups present
277 compared to high DM samples. It has been reported that in presence of low amounts of calcium,
278 there is mono-complexation of Ca^{2+} -ions and interactions seem to be essentially of an anti-
279 cooperative nature (Fang et al., 2008; Lopes da Silva & Rao, 2006) resulting in a weaker
280 network and probably a less compact structure. As the cation concentration increases, pectin
281 forms a stronger network through dimerisation with ion-polymer interactions being essentially
282 cooperative (Lopes da Silva & Rao, 2006) possibly leading to a more compact structure.
283 According to Ngouémazong et al. (2012), at higher Ca^{2+} -concentrations ($R \geq 2.0$), the
284 “improved” network characteristics indicate a conversion from an entangled into a cross-linked
285 network. In fact, an increase in gel stiffness (G') was observed when the Ca^{2+} -ion concentration
286 was increased (increase in R value) until a certain point beyond which no change or a slight
287 decline in gel stiffness was observed (Figure 2B). A drop in gel stiffness can be due to syneresis
288 attributed to the number of cross-links becoming very large compared to the amount of polymer.
289 Next to the elastic modulus (G') of the systems, the viscous modulus G'' was established and
290 results indicated that G' was always higher than the G'' (results not shown) implying that even
291 though some systems may appear as thick liquids (especially at low R values), they should be
292 considered as weak gels. In SBP gels, increasing the cation concentration did not seem to
293 improve network formation and at a given DM, CP exhibited a more cross-linked network than
294 SBP. This observation may be attributed to the steric hindrance of acetyl groups in SBP, which
295 to a certain extent limits the access of Ca^{2+} -ions to a close proximity of two neighbouring

carboxyl groups (Ralet et al., 2003). In fact, Rolin et al. (1998) highlight the presence of O-acetyl groups as one of the reasons for failure of sugar beet pectin to produce reasonably strong gels. The poor gelling of SBP may also be ascribed to its higher neutral sugar content particularly rhamnose, arabinose and galactose (cf. Table 1). The rhamnose inserts on the homogalacturonan chain are said to produce “kinks” in the chain thereby limiting cross-linking. Moreover, the dimensions of the rhamnose molecule are not compatible with the formation of junction zones (Axelos, Lefebvre, Qiu, & Rao, 1991).

3.2.3 Microstructure and gel-stiffness of Fe^{2+} , Zn^{2+} and Mg^{2+} -mediated pectin gels

The micrographs in Figure 3A show gels of 1% pectin solutions at pH 6.2 and R value of 5. From these results, it can be observed that in presence of cations other than Ca^{2+} -ions, pectin also forms gels with a similar network structure as in the case of Ca^{2+} -mediated gels. In presence of Zn^{2+} and Fe^{2+} -ions, pectin seems to form gels with probably a denser and more cross-linked network than in the case with Ca^{2+} -ions. This suggests stronger binding and probably different binding mechanisms. In fact, Assifaoui et al. (2015) shows that zinc is able to bind to hydroxyl groups in addition to carboxylic groups. Zn^{2+} and Fe^{2+} -ions are also reported to possess a high affinity for protein groups. The effect of DM and pectin source on network formation was still evident in these samples with CP 16 gels exhibited a highly cross-linked network. As the DM increased to 35% and 65% (CP 35 and CP 65) less cross-linking was observed as shown in Figure 3A. SBP gels also showed improved network formation probably due to binding via other non-specific interactions to slightly improve gelling. On the other hand, gels with Mg^{2+} -ions exhibited a more open structure with a less intertwined network (Figure 3A) compared to the other gels. According to Rolin et al. (1998), most metal-ions other than monovalent ones work in a similar way as calcium except the Mg^{2+} -ion which is not effective as a pectin cross-linking

agent. However, in these samples (Figure 3A), the effect of DM is still apparent, with gels of CP 16 exhibiting a somewhat closely intertwined fibrous network compared to CP 35 and CP 65. Looking at the rheology of the pectin gels in Figure 3B, the curves of Zn^{2+} and Fe^{2+} -mediated pectin gels were slightly higher or comparable to those of Ca^{2+} -mediated pectin gels while those of Mg^{2+} -mediated pectin-gels were significantly lower ($P<0.05$) than the curves of Ca^{2+} -mediated pectin gels (Figure 1B). To this extent, Zn^{2+} and Fe^{2+} -pectin gels exhibited a slightly higher/comparable gel stiffness to Ca^{2+} -mediated pectin gels while Mg^{2+} -gels showed a lower gel stiffness than Ca^{2+} -pectin gels and these trends correlated with results of cryo-SEM.

Increasing the Zn^{2+} , Fe^{2+} and Mg^{2+} -ions concentration gave rise to gels with a more intertwined fibrous network (supplementary Figure 1) as was observed by increasing Ca^{2+} -ions. These results were complemented by gel stiffness (G') which increased significantly ($P<0.05$) with cation concentration as shown in Figure 3B. Similar to the case of Ca^{2+} -mediated pectin-gels, the stiffness of the gels increased up to a certain extent beyond which no increase or a drop in G' was observed. In Zn^{2+} and Fe^{2+} -mediated pectin-gels, optimum gel stiffness was achieved at R value of ~ 1.5 - 2.5 depending on the DM compared to R value of ~ 3.5 - 4.0 in Mg^{2+} -mediated pectin-gels. From Figure 3B it can be seen that in order to attain a certain gel-stiffness more Mg^{2+} -ions are needed compared to Fe^{2+} and Zn^{2+} -ions which is not surprising considering that Mg^{2+} is a poor cross-linking agent. Finally, it could be concluded that although the type of cation plays a role in determining the final properties of gels, the DM of pectin and source of the pectin are vital for gel formation.

3.2.4 Effect of pH on the microstructure and rheology of cation-mediated CP and SBP-gels

According to Lopes da Silva and Rao. (2006), although pH is not so critical in development of low DM pectin-gels as it is for high DM pectin-gels, it has a significant effect on the final

properties of the gel. Therefore, the effect of pH on gel properties was examined. Figure 4A shows micrographs of Ca^{2+} -pectin gels of 1% pectin solutions at pH 3.5 and pH 6.2. From these results, it can generally be observed that at a lower pH (pH 3.5), pectin gels exhibit a less intertwined fibrous network compared to those at higher pH (pH 6.2). This may be attributed to the lower number of ionised carboxyl group given that pH 3.5 is approximately equal to the pKa value of pectin (3.38-4.10) (Moore,1985), a pH at which about 50% of chargeable groups are ionised. CP 16 and CP 35 showed a more cross-linked network while limited cross-linking (if any) was observed in CP 65 and CP 95-pectin gels (results not shown). In the high DM samples, at this pH probably the number of ionised carboxylic groups was insufficient to initiate and promote interactions. Additionally, SBP 35 and SBP 60 exhibited limited intertwining at this pH (as shown in supplementary Figure 2).

As the pH was increased to 6.2, gel microstructures became more compact and exhibited a more intertwined fibrous network especially CP 16 and CP 35 as shown in Figure 4A. As pH 6.2 is above the pKa value of pectin (Rolin, Nielsen, & Glahn, 1998), there was almost complete ionisation of non-methylesterified carboxyl groups there by facilitating increased cross-linking. In fact, Ralet et al. (2003) reports a greater charge density when the pH of a solution is above the apparent pKa value of pectin. Furthermore, according to Lopes da Silva & Rao (2006), binding of Ca^{2+} -ions increases with pH, showing a maximum level at pH 5-7.5. Even in the case of CP 65, a fibrous network is evident at this pH probably due to an increase in the number of ionised carboxyl groups. For a given DM, the fibrous network exhibited by CP-gels was more intertwined than that of SBP-gels.

From the results of rheology in Figure 4B, it was clear that gels at pH 3.5 were significantly weaker ($P < 0.05$) than those at pH 6.2 evidenced by lower G' values. These results correlated

with Cryo-SEM images in Figure 4A where increased intertwining of the gel-fibrous network was observed with increasing pH. An appreciable increase in gel stiffness ($P < 0.05$) was observed for CP 65-gels with increasing pH which is related to the report of Lopes da Silva and Rao, (2006) stating that development of high DM pectin gels is largely pH dependent which controls the number ionised carboxyl groups. In conclusion, results of gel- microstructure were complemented by rheology results. Considering that for a given DM and pectin source equal amounts of Ca^{2+} -ions were added to the gels at pH 3.5 and pH 6.2, it can be concluded that, at low pH, more calcium is needed to induce gelation than at neutral pH.

3.2.5 Effect of pectin concentration on the microstructure of CP and SBP-gels

According to Lopes da Silva & Rao, M. A. (2006), increasing pectin concentration led to an increase in gel stiffness (explored for pectin concentrations of 0.5%-2%). In this research, this observation was demonstrated by increasing the pectin concentration at a fixed cation concentration. From Figure 5A, it can generally be observed that, increasing the pectin concentration from 1% to 2% results in gels with a denser fibrous network. Theoretically, by doubling the pectin concentration, the number of ionised carboxylic groups doubles thereby increasing the ion-binding capacity of pectin as well cation sensitivity. In fact, increasing the pectin concentration also led to significant increase in gel stiffness ($P < 0.05$) and this increase was pronounced in CP 65, a higher DM sample (Figure 5B). This could be attributed to a sufficient number of carboxyl groups in 2% pectin gels which initiated and promoted network formation compared to 1% CP 65-gels. In addition, the extent of shear thinning exhibited by 2% pectin gels was less than that of 1% pectin-gels as shown in Figure 5C, an indication of a stronger network in the 2% pectin-gels.

3.2.6 Microstructure of CP and SBP gels at a fixed cation concentration

Figure 6A shows results obtained for pectin gelled by addition of a fixed amount of Ca^{2+} -ions to 2% pectin solutions with different DMs. This was aimed at exploring the sensitivity of pectin to gelling. Irrespective of the type of cation present, the fibrous network exhibited by low DM pectin gels was more intertwined than that of high DM pectin. It can be seen that when a similar concentration of cation is added to the sample, the factor limiting gel formation would most probably be the number of ionised carboxyl groups present. This infers that low DM samples (which possess more ionised carboxyl groups) will cross-link to a greater extent than high DM samples. For a given DM, CP was more sensitive to Ca^{2+} -gelling compared to SBP (for instance CP 35 compared to SBP 35) which is not surprising considering various reports of the poor gelling properties of SBP attributed to its structure. Results of rheology in Figure 6B complemented observations of Cryo-SEM. Gel stiffness increased significantly ($P < 0.05$) with decreasing DM and at given DM, CP gels were significantly ($P < 0.05$) stiffer than SBP gels.

Conclusion

The structure-function relationship of pectin in pectin gels is commonly established by relating the rheological behaviour of the gels to the physiochemical structure of the pectin. In this research, the underlying gel-microstructure/network was visualised using cryo-scanning electron microscopy (Cryo-SEM) and related to the pectin physiochemical structure as well as rheological behaviour in order to provide more insight into the structure-function relationship. The microstructure of conventional pectin gels (Ca^{2+} -mediated pectin gels) as well as non-conventional pectin gels (Zn^{2+} , Fe^{2+} and Mg^{2+} -mediated pectin gels) of citrus and sugar beet pectin was explored. Cryo-SEM images revealed that the type of cation added influences gel-microstructure with Fe^{2+} , Ca^{2+} and Zn^{2+} -gels showing a highly intertwined network compared to

Mg²⁺-gels. Next to type of cation, pectin properties including pectin DM and pectin source influenced network formation/gel-microstructure. As DM decreased, increased intertwining of the fibrous network was observed and at a given DM, the network exhibited by the more linear-less acetylated pectin (CP) was more intertwined than that of highly branched-more acetylated pectin (SBP). Additionally, by increasing the pectin concentration, pH and cation concentration, the gel-network became more intertwined. Finally, as analysis of the rheological behaviour revealed that increases in gel-stiffness were complemented by increased network formation, a correlation between rheology and gel-microstructure could be concluded. As such, Cryo-SEM can be used in combination with quantitative measurements to provide more information on product properties.

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517
518

Figure captions

Figure 1. A) Representative images of the microstructure of 1% CP and SBP solutions at pH 6.2 with no added cations. Scale bars = 1 μm . B) The flow behaviour of the pectin solutions at different shear rates.

Figure 2. Representative results of A) microstructure of Ca^{2+} -mediated pectin gels of 1% CP and SBP with different degrees of methylesterification at various R-values. Scale bars = 1 μm . B) The associated gel stiffness (G') at 1 rad/s.

Figure 3. Influence of Zn^{2+} , Fe^{2+} and Mg^{2+} -ions on gel properties. A) Representative images of the microstructure of Zn^{2+} , Fe^{2+} and Mg^{2+} -mediated pectin gels of 1% pectin samples at an R-value of 5. Scale bars = 1 μm . B) The evolution of gel stiffness (G') at 1 rad/s with increasing R value.

Figure 4. Effect of pH on the final properties of Ca^{2+} -mediated pectin gels of 1% pectin with different degrees of methylesterification. A) Microstructure of the gels at pH 3.5 and pH 6.2, at an R-value of 5. Scale bars = 1 μm . B) The elastic (G') and viscous (G'') behaviour of the gels.

Figure 5. Ca^{2+} -mediated pectin gels of 1% and 2% pectin (CP 16 and CP 65) at an a R-value of 1. A) The microstructure of the gels, scale bars = 1 μm , B) the associated elastic (G') and viscous (G'') behaviour, and C) the apparent viscosity of the gels at different shear rates.

Figure 6. Gelling ability of CP and SBP pectin with comparable degrees of methylesterification at a given cation concentration. A) Microstructure of the 2% pectin gels mediated by Ca^{2+} , Zn^{2+} and Fe^{2+} -ions. Scale bars = 1 μm . B) The associated elastic (G') and viscous (G'') behaviour.

Figure 1

A

1% pectin

B

Figure 2

A

Ca²⁺-mediated pectin gels (1% pectin)

B

Figure 3

A

1% pectin

B

Figure 4

A

Ca²⁺-mediated pectin gels (1% pectin)

B

Figure 5

A

Ca²⁺-mediated pectin gels (1% and 2% pectin)

B

C

Figure 6

A

B

Pectin property	Citrus pectin	Sugar beet pectin
	(CP 16, CP 35, CP 65 and CP 95)	(SBP 35 and SBP 60)
Pectin-related sugars		
(mg/g pectin)		
Galacturonic acid	730.5 ± 21.2	550.1 ± 21.0
Galactose	48.5 ± 1.05	95.7 ± 0.84
Arabinose	1.3 ± 0.38	28.5 ± 0.55
Rhamnose	8.8 ± 0.46	53.5 ± 0.71
Xylose	4.9 ± 0.85	3.0 ± 0.42
Fucose	0.2 ± 0.06	0.8 ± 0.06
Other sugars		
Mannose	1.7 ± 0.26	4.2 ± 0.23
Glucose	4.4 ± 0.46	6.8 ± 0.86
Molar mass (kDa)	43.0 ± 1.27	778.0 ± 20.7
Protein content (%)	1.6 ± 0.03	3.5 ± 0.08
Degree of feruloylation (%)	0.7 ± 0.03	3.3 ± 0.14
Degree of acetylation (%)	2.5 ± 0.29	20.7 ± 0.52

Table 1: Results of characterisation of citrus pectin and sugar beet pectin samples with the associated standard deviations.

Sample (1% pectin, w/v-pH 6.2)	Viscosity (Pa.s) at 1 s⁻¹
CP 16	262 x 10 ⁻⁴
CP 35	219 x 10 ⁻⁴
CP 65	141 x 10 ⁻⁴
CP 95	41.4 x 10 ⁻⁴
SBP 35	152 x 10 ⁻⁴
SBP 60	126 x 10 ⁻⁴

Table 2: The apparent viscosity of 1% pectin solutions of different degrees of methylesterification at pH 6.2 and shear rate of 1 s⁻¹.

Supplementary Data

1% pectin-pH 6.2

Supplementary Figure 1: Microstructure of representative images of Fe²⁺ and Zn²⁺-mediated pectin gels of CP 16, CP 35 and CP 65 at R values of 1 and 5. Pectin solutions (1%, w/v) at pH 6.2 were used. Scale bars = 1 μm.

Ca²⁺-mediated pectin gels (1% pectin)

Supplementary Figure 2: Microstructure of representative images of Ca²⁺-mediated pectin gels of SBP 35 and SBP 60 (1% pectin solutions, w/v) at pH 3.5 and 6.2, and an R value of 5. Scale bars = 1 μm.