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Rheological properties of Ca^{2+}-gels of partially methylesterified polygalacturonic acid: Effect of "mixed" patterns of methylesterification

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ABSTRACT

Subjecting homogalacturonan to a combination of processes which modify the extent of pectin's methylesterification results in the generation of distinct patterns of methylesterification on the polymer. In this study, the influence of these "mixed" patterns of methylesterification on the rheological characteristics of Ca^{2+}-gels is extensively examined, using partially methylesterified poly-D-galacturonic acid (mPGA) as a model for homogalacturonan. The uncontrolled pattern of methylesterification induced on mPGA was combined with that resulting from subsequent controlled chemical or enzymatic de-esterification. Afterwards, the produced mPGAs were characterised in terms of degree and pattern of methylesterification. The latter structural feature was quantified as "absolute degree of blockiness" through enzymatic fingerprinting. Characterised mPGAs were used for the preparation of gels with various calcium ion (Ca^{2+}) concentrations. All Ca^{2+}-pectin systems formed "true" gels with a strong elastic behaviour, but vary in gel stiffness. The gels' rheological characteristics not only depend on the extent of mPGA de-esterification, but also on the "mixed" patterns of methylesterification. The experimental results also indicate that extremely short non-cooperative junction zones contribute to the stiffness of Ca^{2+}-mPGA gels.

1. Introduction

Pectin is a family of plant cell wall polysaccharides consisting mainly of linear homogalacturonan (HG) and branched rhamnogalacturonan domains. HG is a homopolymer of α(1-4)-linked D-galacturonic acid (GalA) residues, carrying methylesters at the C6 position of certain monomers (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). This remarkable feature of HG confers a specific degree of methylesterification (DM) to pectin and controls numerous functional properties of this naturally occurring polymer. Generally, pectins of low DM are able to form gels in the presence of divalent cations such as calcium ions (Ca^{2+}) and optimally, at high pH (well above 3.5, the acid dissociation constant of GalA) (Powell, Morris, Gidley, & Rees, 1982; Thibault & Ralet, 2003). Ca^{2+}-pectin gels are formed according to the so-called "egg-box" model, in which stretches of non-methylesterified GalA (NM-GalA) residues of pectin's HG chains are ionically cross-linked through Ca^{2+} bridges. Both the amount of Ca^{2+} ions and the structural features of pectin, such as the DM and pattern of methylesterification (PM) influence the mechanical (rheological) properties of the formed gels (Cárdenas, Goycoolea, & Rinaudo, 2008; Fraeye et al., 2009; Guillotin, Boulenguer, Mazoyer, Schols, & Voragen, 2005; Ngouémazong, Tengweh, et al., 2012; Powell et al., 1982; Ström et al., 2007; Willats et al., 2001). This gelling property renders both *in planta* and extracted pectins valuable functional polymers (Endress, Mattes, & Norz, 2006; Van Buggenhout, Sila, Duvetter, Van Loey, & Hendrickx, 2009).

The structure of pectin's HG can be modified through the controlled hydrolysis of methylesters. This de-esterification is carried out on extracted pectin to produce tailored polymers suitable as functional ingredients, for use in specific applications (Endress et al., 2006; May, 1990). Pectin modifications can also be performed *in situ* as a means of "engineering" desirable texture of

Abbreviations: DB_{abs}, absolute degree of blockiness; DM, degree of methylesterification; GalA, D-galacturonic acid; HG, homogalacturonan; mPGA, methylesterified poly-D-galacturonic acid; C-mPGA, chemically de-esterified mPGA; F-mPGA, fungal PME de-esterified mPGA; P-mPGA, plant PME de-esterified mPGA; NM, non-methylesterified; NM-MDT-GalA, non-methylesterified mono-, di- and tri-galacturonic acid; PDP, partially de-esterified pectins; PG, polygalacturonase; PGA, poly-D-galacturonic acid; PM, pattern of methylesterification; PME, pectin methylesterase.

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processed fruits and vegetables (Van Buggenhout et al., 2009). The methylesters of HG can be hydrolysed either chemically using NaOH or enzymatically using pectin methylesterase EC 3.1.1.11 (PME) of plant or fungal origin. NaOH de-esterification has been reported to yield polymers with a completely random distribution pattern of NM-GalA residues. Fungal PME de-esterification results in polymers with random distribution of stretches of NM-GalA residues (referred to as NM-GalA blocks) whereas plant PME produces large NM-GalA blocks on HG chains in a sequential fashion (Limberg et al., 2000).

In practice, the PMs often exhibited by pectins are not purely the result of one of the abovementioned de-esterification methods. In fact, the DM of extracted pectins varies with the production process (Koubala et al., 2008). The de-esterification that occurs during pectin extraction results in the production of polymers with an uncontrolled PM. In view of producing low-methylesterified pectins, subjecting extracted pectin to controlled modification via enzymatic (plant or fungal PMEs) or chemical de-esterification may finally result in polymers with distinct PMs. The PM resulting from a combination of processes is described as "mixed" PM. Since the structure of pectin controls Ca^{2+}-pectin gel characteristics, gels prepared from pectins with "mixed" PMs may reveal mechanical properties which are different from the usual characteristics of gels prepared from purely blockwise or randomly de-esterified pectins.

Therefore, the present work aims at studying the effects of "mixed" patterns of methylesterification on the rheological properties of Ca^{2+}-pectin gels. In our approach, partially methylesterified poly-D-galacturonic acid is used as a model for pectin. Poly-D-galacturonic acid (PGA) is a linear homopolymer consisting of α(1-4)-linked D-galacturonic acid (GalA) residues. Structurally, this polymer resembles pectin's HG, with the difference that a number of the GalA residues of the naturally occurring polymer are methylesterified. Introducing methylesters on PGA yields partially methylesterified PGA, referred to as mPGA. This partial esterification of PGA may generate distribution pattern of methylesters on the polymer in an uncontrolled way, similar to that of extracted pectins. Hence, mPGA represents a good model for pectin's HG. Moreover, the use of a linear polymer enables to exclude any influence of pectins' side chains on gel characteristics, particularly as neutral sugar side chains are reported to improve the rheological characteristics of Ca^{2+}-pectin gels (Ngouémazong, Kabuye, et al., 2012). The strategic choice of mPGA will enable to derive correlations between "mixed" patterns of methylesterification and the properties of Ca^{2+}-gels.

Consequently, PGA was partially esterified to produce mPGA, which was subjected to chemical or enzymatic de-esterification. Ca^{2+}-mPGA gels were prepared at various Ca^{2+} concentrations. Subsequently, the rheological characteristics of the gels were assessed and related to the structural features of the polymers.

2. Materials and methods

2.1. Materials

PGA (product number 81325, of average molar mass estimated to be 58.39 ± 4.2 kDa) was purchased from Sigma (Belgium). A commercial liquid preparation of recombinant *Aspergillus aculeatus* PME purchased from Novozymes (Denmark) was purified by gel filtration chromatography (Duvetter et al., 2006). Plant PME was extracted from carrot (*Daucus carota var. nantes* of Belgian origin) and purified using affinity chromatography (Jolie et al., 2009). Pure endo-polygalacturonase EC 3.2.1.15 (endo-PG) from *Kluyveromyces fragilis* was kindly provided by the Laboratory of Food Chemistry of Wageningen University. All chemicals used were of analytical grade.

2.2. Methylesterification of poly-D-galacturonic acid

PGA was chemically methylesterified based on the method of Zachariassen et al. (2006). PGA, methanol and thionyl chloride (in the ratio of 1:5:1) were allowed to react at 3 °C and under continuous stirring for 7 days. The produced partially methylesterified PGA was recovered by vacuum filtration and methanol washing. The washed mPGA was then dissolved and extensively dialysed against demineralised water, at 4 °C. Dialysis ensured the complete removal of free methanol. Subsequently, the dialysed solution was lyophilised and stored above P_2O_5. The produced mPGA was encoded M53 (where 53 represents the extent of methylesterification of the polymer) and used as the starting material for de-esterification.

2.3. Partial de-esterification of mPGA

A controlled partial de-esterification of M53 was carried out using plant (*Daucus carota*) pectin methylesterase (PME), fungal (*A. aculeatus*) PME or chemical saponification (NaOH) to yield P-, F- or C-mPGAs with various degrees and patterns of methylesterification. P-mPGA samples were produced at pH 7.0, while the production of F- and C-mPGAs was carried out at pH 4.5 and 11.0, respectively. Detailed de-esterification procedures have been described previously (Fraeye et al., 2009; Ngouémazong et al., 2011).

2.4. Characterisation of partially de-esterified mPGA

Partially de-esterified mPGA samples were characterised in terms of DM and PM, using the methods described by Ngouémazong et al. (2011) for pectin characterisation.

DM was calculated as the ratio of the molar amount of methanol released to the molar amount of GalA per gram sample (on dry basis) and expressed in percentage. After complete hydrolysis of methylesters in all mPGA samples (including the parent mPGA sample, M53), the methyl group content (as methanol) was estimated based on the colorimetric method of Klavons and Bennett (1986). Following a sulfuric acid hydrolysis of M53, its GalA concentration was determined by the colorimetric m-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973). The GalA content of the mPGA samples (P-, F- and C-mPGAs) was not determined analytically; rather it was calculated, as earlier explained by Ngouémazong et al. (2011). Some theoretical assumptions were made in order to develop a formula for the estimation of the GalA content of partially de-esterified mPGA samples. During de-esterification, the amount of methyl groups of the parent M53 is supposed to decrease while the ratio of the amount (dry basis) of GalA to the amount (dry basis) of other constituents (generally impurities) remains constant in both M53 and a de-esterified mPGA. In the current work, as the moisture content of all samples was checked and found to be the same, the varying parameter between a specific weight of M53 and that of a de-esterified mPGA was the contribution of methyl groups to the weight of both samples. Hence, knowing the methyl group content of M53, the GalA content of M53 as well as the methyl group content of a de-esterified mPGA, its GalA content was deduced as follows:

$$GalA_{(mPGA)} = \frac{GalA_{(M53)} \times (1 - CH_{3(mPGA)})}{1 - CH_{3(M53)}} \text{(Ngoué mazong et al., 2011)}$$

where $GalA_{(mPGA)}$: g GalA/g mPGA; $GalA_{(M53)}$: g GalA/g M53; $CH_{3(mPGA)}$: g CH_3/g mPGA and $CH_{3\ (M53)}$: g CH_3/g M53.

The PM, quantified as absolute degree of blockiness (DB_{abs}), was determined after an enzymatic fingerprinting of the partially

de-esterified P-, F- and C-mPGA. In short, mPGA samples were subjected to an extensive enzymatic depolymerisation using endo-polygalacturonase (endo-PG) from *K. fragilis*, thereby producing non-methylesterified galacturonides, as non-methylesterified mono-, di- and tri-GalA (NM-MDT-GalA) as well as methylesterified oligomers (Daas, Arisz, Schols, De Ruiter, & Voragen, 1998). The HPAEC determination of each NM-galacturonide released enabled to calculate the total amount of NM-MDT-GalA released (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999). DB_{abs} was calculated as the molar ratio of the total NM-MDT-GalA released to the total GalA of the polymer as follows:

$$DB_{abs}(\%) = \frac{[\text{mono-GalA}] + 2 \times [\text{di-GalA}] + 3 \times [\text{tri-GalA}]}{GalA_{(mPGA)}} \times 100 \quad \text{(Strö m et al., 2007)}$$

where [mono-GalA], [di-GalA] and [tri-GalA] were concentrations of non-methylesterified mono-, di- and tri-GalA released, respectively, and expressed in mol/g sample; $GalA_{(mPGA)}$ represented the GalA content of a de-esterified mPGA samples, expressed in mol/g sample. Both DM and DB_{abs} were determined in duplicate.

The HPSEC analysis of endo-PG hydrolysed mPGAs enabled to determine the intermolecular PM of mPGAs, via the assessment of molar mass distribution profiles (Duvetter et al., 2006).

2.5. Preparation of Ca^{2+}-mPGA gels

Ca^{2+}-mPGA gels were prepared using ~1.74 (1.71–1.77% w/v) mPGA solutions (i.e. exactly 1.68% w/v GalA, as the GalA content of mPGAs is about 95%) at pH 6.0 (±0.05), as described by Ngouémazong, Tengweh, et al. (2012). Gels were produced at various Ca^{2+} concentrations, expressed as the ratio $R = 2[Ca^{2+}]/[COO^-]$. The *R*-value of the mPGA gels was varied between 0.25 and 3.0. A technique involving the combination of mild heating (50 °C) and diffusion of Ca^{2+} through microlitres of the mPGA solution was used for Ca^{2+}-mPGA gel preparation, as described by Ngouémazong et al. (2011). Ca^{2+}-mPGA gels were prepared directly on the preheated lower plate of a stress-controlled Physica MCR 501 rheometer (Anton Paar, Austria) using a few microliters of preheated mPGA and $CaCl_2$ solutions.

2.6. Small-amplitude oscillatory shear tests

Once a Peltier-controlled hood was placed over the sample loaded on the rheometer (time zero of the experiment), the Ca^{2+}-mPGA mixture was allowed to equilibrate for 10 min, after which it was cooled from 50 °C to 20 °C within 1 h (0.5 °C/min). The gel was subsequently let to evolve for 5 h at 20 °C. Preliminary stress sweep tests (at 1 rad/s) were carried out on a number of Ca^{2+}-mPGA gels in order to define the linear viscoelastic region of the gels, in which the viscoelastic response of the sample (storage modulus (G') and loss modulus (G'')) is independent of the applied stress amplitude. All subsequent oscillatory shear tests were carried out within this linear region, thereby excluding effects of shear-induced gelation or structure breakdown. During the cooling step and the 5 h isothermal (at 20 °C) period, a time sweep test was performed at 1 rad/s in order to monitor the structure development of the gels. At the end of gel formation, a frequency sweep test (0.1–10 rad/s) enabled to investigate the frequency dependence of the gel moduli and the gel stiffness (storage modulus at constant angular frequency (1 rad/s)). Although the gel preparation method and rheological test results have been reported to be very reproducible (Doungla et al., 2009), in the present study, most gel samples were analysed in duplicate, on freshly prepared samples.

3. Results and discussion

3.1. Structural characteristics of partially de-esterified mPGAs

The methylesterification of poly-D-GalA yielded the parent polymer for de-esterification, M53, with DM ~53%. M53 was partially de-esterified using plant (*Daucus carota*) PME, fungal (*A. aculeatus*) PME or chemical saponification to produce P-, F- or C-mPGAs of various DM. None of the mPGAs showed a shift in elution time as compared to PGA on HPSEC chromatograms (results not shown), indicating that the production processes did not cause any depolymerisation of the polymers.

Following an extensive hydrolysis of the produced mPGAs using *K. fragilis*' endo-PG, the molar concentrations of NM-galacturonides (as mono-, di- and tri-GalA) released were determined both in absolute amounts and proportions. Subsequently, DB_{abs} was calculated as formulated in Section 2.4. An assessment of the proportions of the NM-galacturonides released as well as the DB_{abs} enabled to examine the evolution of NM-GalA blocks in terms of the size and the number of the blocks occurring in the polymer at successive stages of de-esterification. In addition, HPSEC chromatography analysis of the endo-PG digested samples enabled to assess the intermolecular PM of various mPGAs.

3.1.1. Assessing the proportions of non-methylesterified mono-, di- and tri-GalA released from mPGAs

The proportions of mono-, di- and tri-GalA released as a function of DM for the three types of mPGAs is displayed in Fig. 1. Regardless of the extent and method of de-esterification, all mPGAs (including the parent polymer, M53) revealed similar proportions of the galacturonides, with tri-GalA showing the greatest contribution (~60%) and mono- and di-GalA displaying the smallest (both ~20%). Interestingly, these proportions were comparable to those obtained after endo-PG digestion of PGA (DM = 0%). This observation indicates that all mPGAs, even those with high DM, bear NM-GalA blocks of sizes large enough to allow endo-PG digestion to be similar to that of the ester-free polymer, PGA. Based on the mode of action of *K. fragilis* endo-PG as proposed by Pasculli, Geraeds, Voragen, and Pilnik (1991), the release of NM-tri-GalA requires the presence of NM-GalA blocks with at least seven contiguous NM-GalA residues (Ngouémazong, Jolie, et al., 2011). This implies that all mPGAs are characterised by the presence of blocks with at least seven NM-GalA residues. The comparable proportions of NM-MDT-GalA displayed in all mPGAs results from the presence of large NM-GalA blocks on M53, meaning that the methylesterification step imposed some common features in all mPGAs. Similar proportions of 60% for NM-tri-GalA and 20% for both NM-di- and mono-GalA have been reported for plant PME de-esterified pectins of comparable DM, whereas F- and C-pectins revealed these proportions only at lower DM values (i.e. ≤36%) (Ngouémazong et al., 2011).

3.1.2. Assessing the changes in DB_{abs} upon partial de-esterification of mPGA

The determination of the molar concentrations of non-methylesterified mono-, di- and tri-GalA released also enabled the quantification of polymers' PM as DB_{abs}. Fig. 2 shows DB_{abs} as a function of DM for the mPGA samples obtained by the three different de-esterification methods. This figure also illustrates the DB_{abs} values of partially de-esterified pectin samples produced from a high-methylesterified pectin, either by plant PME, fungal PME or chemical de-esterification (P-, F- and C-pectins) (Ngouémazong et al., 2011).

The parent mPGA, M53, showed a relatively high DB_{abs} value (~18%), indicating that the total amount of NM-MDT-GalA released from the polymer was quite large. This observation confirms the effective presence of large NM-GalA blocks on M53. At a DM

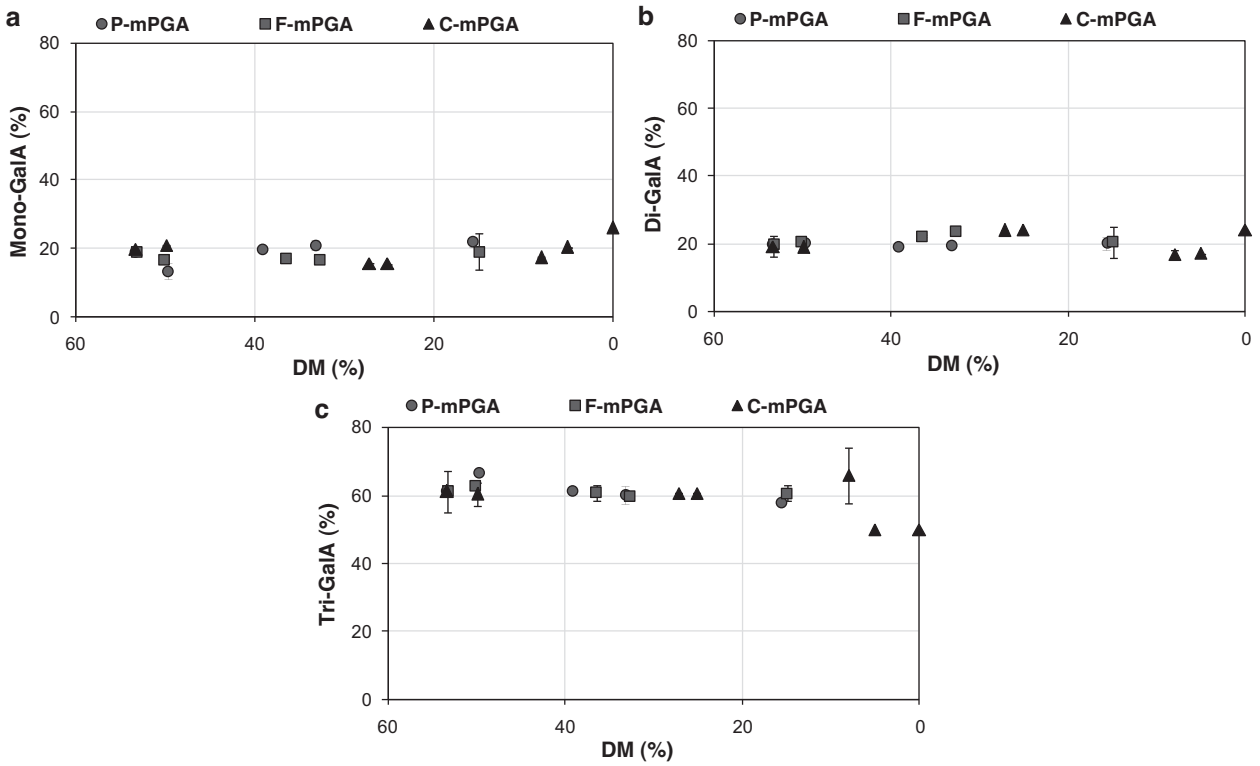

Fig. 1. Proportions of mono- (a), di- (b) and tri-GalA (c) released upon endo-PG digestion of mPGAs, as a function of the DM. The DM of 0% is commercial PGA that did not undergo methylesterification.

similar to that of M53, P- and C-pectins revealed higher and lower DB_{abs} values, respectively. Conversely, a F-pectin of similar DM as M53 displayed a DB_{abs} value comparable to that of M53, thereby revealing the apparent intermediate (neither completely random nor completely blockwise), though uncontrolled, PM of M53.

Subsequent partial de-esterification of M53 was accompanied, in P-mPGAs, by a gradual increase in DB_{abs}. Actually, this increasing trend was rather similar to the one displayed by P-pectins. This increase in DB_{abs} with decreasing DM indicates an immediate (early stages of de-esterification) and progressing increase in the total NM-galacturonides released from P-mPGAs. This suggests that plant PME de-esterification causes a rapid increase of the size of the NM-GalA blocks already present on M53. Besides, an increase in DB_{abs} may also result from an increase in the number of large NM-GalA blocks in the produced P-mPGAs. Therefore, P-mPGAs are likely characterised by the occurrence of large NM-GalA blocks, of which the size and/or number increase when de-esterification proceeds. This implies a blockwise distribution of NM-GalA residues.

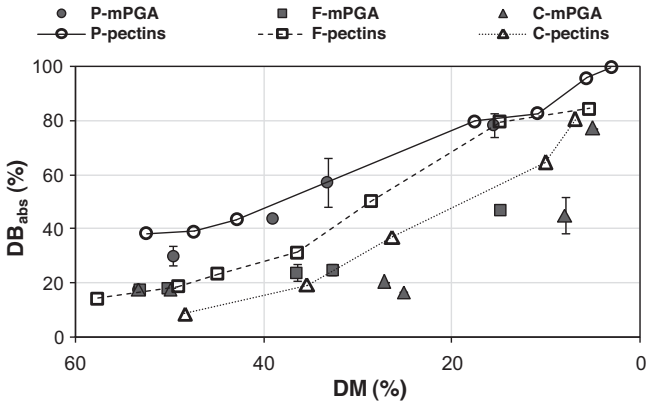

Fig. 2. DB_{abs} (±standard deviation) as a function of DM for the mPGA samples (filled symbols) obtained by chemical methylesterification followed by de-esterification. Comparison with partially de-esterified pectin samples (open symbols) produced by either plant PME, fungal PME or chemical de-esterification.

C-mPGAs, produced after NaOH saponification of the parent M53 showed rather constant values of DB_{abs} (i.e. ≤20%), for DM down to at most ~27%. For DMs between 53 and 27%, the steady DB_{abs} values indicate that comparable amounts of NM-galacturonides were released from different C-mPGAs, independent of DM. This suggests that decreasing the DM of M53 by ~50% (i.e. to DM ~ 27%) through chemical de-esterification resulted in the formation of a high number of very short NM-GalA blocks (which could not enable the release of any NM-galacturonide by *K. fragilis* endo-PG), in addition to the already existing large blocks originating from partial methylesterification. Since a sequence of five contiguous NM-GalA residues is required for the release of a NM-mono-GalA (Daas et al., 1999; Pasculli et al., 1991), it is suggested that in C-mPGA of DM greater than 27%, de-esterification produced NM-GalA blocks with less than five GalA residues. At low values of DM (i.e. <27%), a steep increase of DB_{abs} was displayed, which corresponds to an increase in the size of the existing NM-GalA blocks and, thus, a concomitant increase in the number of large blocks. In pectins, the presence of short NM-GalA blocks similar to those observed in C-mPGAs of DM ≥27% was reported earlier for the polymers produced at the early stages of chemical de-esterification (decrease of initial DM by about 25%) (Ngouémazong et al., 2011).

In F-mPGAs, the trend in the evolution of DB_{abs} with decreasing DM was intermediate between that of P-mPGA and C-mPGA, indicating the release of relatively larger amounts of NM-galacturonides and thus the presence of relatively larger and/or more NM-GalA blocks on F-mPGAs as compared to C-mPGAs. However, at early stages of de-esterification, DB_{abs} remained rather constant up to DM of approximately 33%, suggesting the presence of some short NM-GalA blocks with less than five GalA residues.

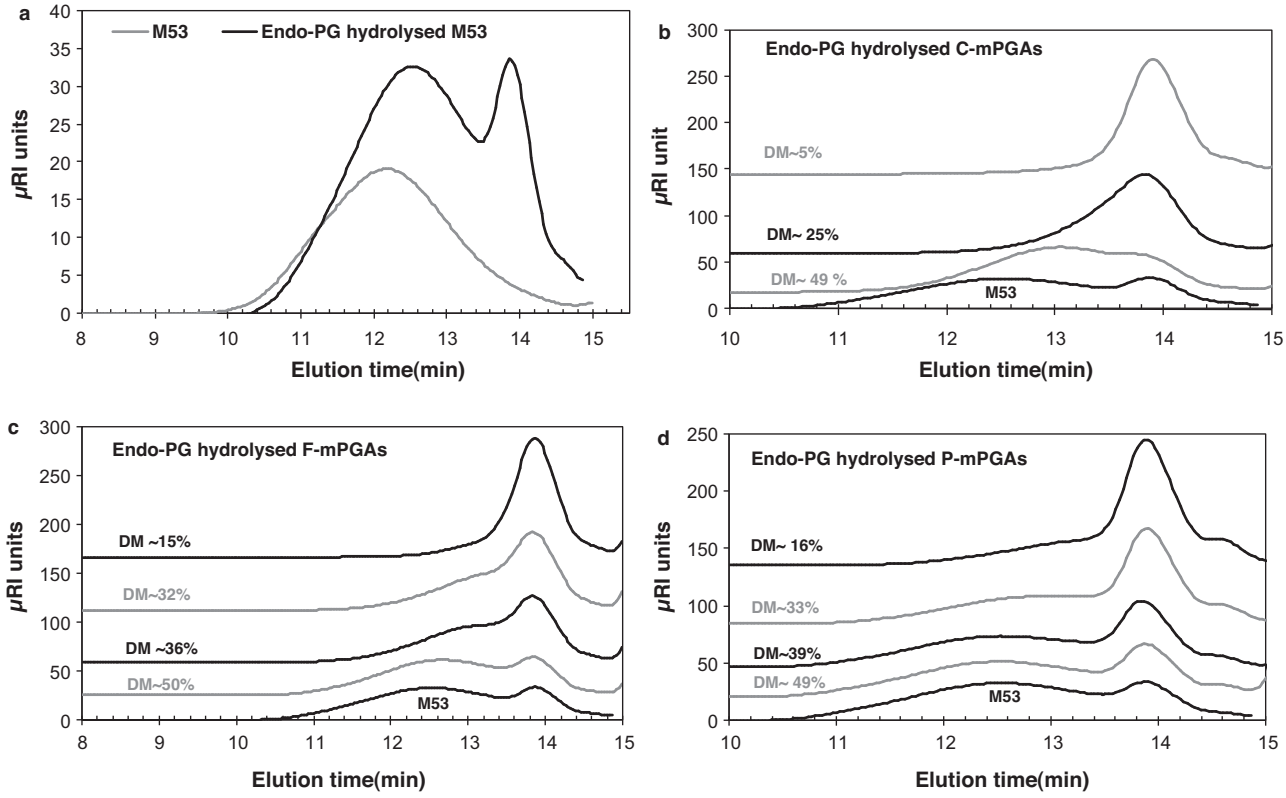

Fig. 3. Molar mass distribution profiles of (endo-PG digested) mPGAs: (a) undigested and digested M53, endo-PG hydrolysed (b) C-mPGAs, (c) F-mPGAs and (d) P-mPGAs.

Unlike C-mPGAs, an increase in DB_{abs} was displayed in F-mPGAs of DM greater than 33%. The lower DB_{abs} of C-and F-mPGA as compared to C- and F-pectin results from the lower DM of M53 (~53%) as compared to the high starting DM of M94 (~94%) for pectins coupled with the formation of short NM-GalA blocks at early stages of both de-esterifications, irrespective of the DM of the starting material.

To summarise, the pattern of methylesterification resulting from the partial chemical esterification of PGA (DM ~53%) followed by successive chemical or fungal PME de-esterification probably exhibits the occurrence of both large and short NM-GalA blocks (with less than five GalA residues) in polymers of DM down to at most 27% or 33% for C- or F-mPGA, respectively. This implies the combination of a blockwise and random distribution of NM-GalA residues. C- and F-mPGAs with low DM reveal polymers with NM-GalA blocks of increased size.

3.1.3. Assessing the intermolecular pattern of methylesterification of mPGAs

The molar mass distribution profiles of endo-PG digested samples are shown in Fig. 3 and enable to assess the intermolecular PM of mPGAs. The starting sample M53 revealed polymeric populations of high and low average molar masses, of which the former is abundant as compared to the latter (Fig. 3a). In addition, the polymeric population of high molar mass showed a slight shift in elution time, when compared to the undigested M53. These observations indicate that most (if not all) M53 molecules were partially depolymerised by endo-PG. This suggests that M53 molecules carried methylester blocks which hindered their complete degradation by endo-PG. The presence of fully methylesterified mPGA chains, or chains with short NM-GalA blocks (with less than four NM-GalA residues), would have resulted in the elution of a polymeric population of similar average molar mass as the undigested M53. Moreover, the small amount of low molar mass polymeric population suggests that these oligomers are degradation products of partially methylesterified mPGA chains. Any depolymerisation of (completely) non-methylesterified mPGA chains would have resulted in higher amounts of oligomers (as endo-PG action is not hindered). Therefore nearly all M53 molecules are partially methylesterified.

Upon de-esterification, the different types of mPGAs (P-, F- and C-mPGAs) showed some differences in the intermolecular PM. While F- and C-mPGAs presented a gradual shift in elution time as sample DM was decreased, P-mPGAs revealed similar distribution profiles as the digested M53 (Fig. 3b, c and d, respectively). However, in P-mPGAs, as samples' DM was decreased, the high polymeric population continuously decreased while the population of lower molar masses increased. These observations indicate that,

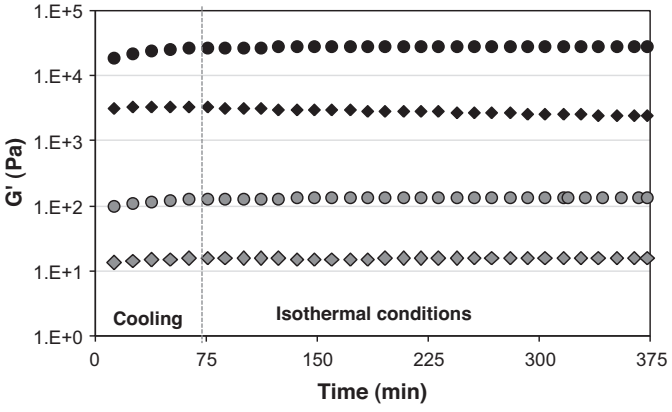

Fig. 4. G' as a function of time for Ca^{2+}-mPGA gels at low Ca^{2+} concentration (◊, $R = 0.25$) and high Ca^{2+} concentration (○, $R = 3.0$). The grey symbols represent M53 gels while black symbols correspond to C-mPGA (DM ~ 5%) gels. Tests were performed at 1 rad/s.

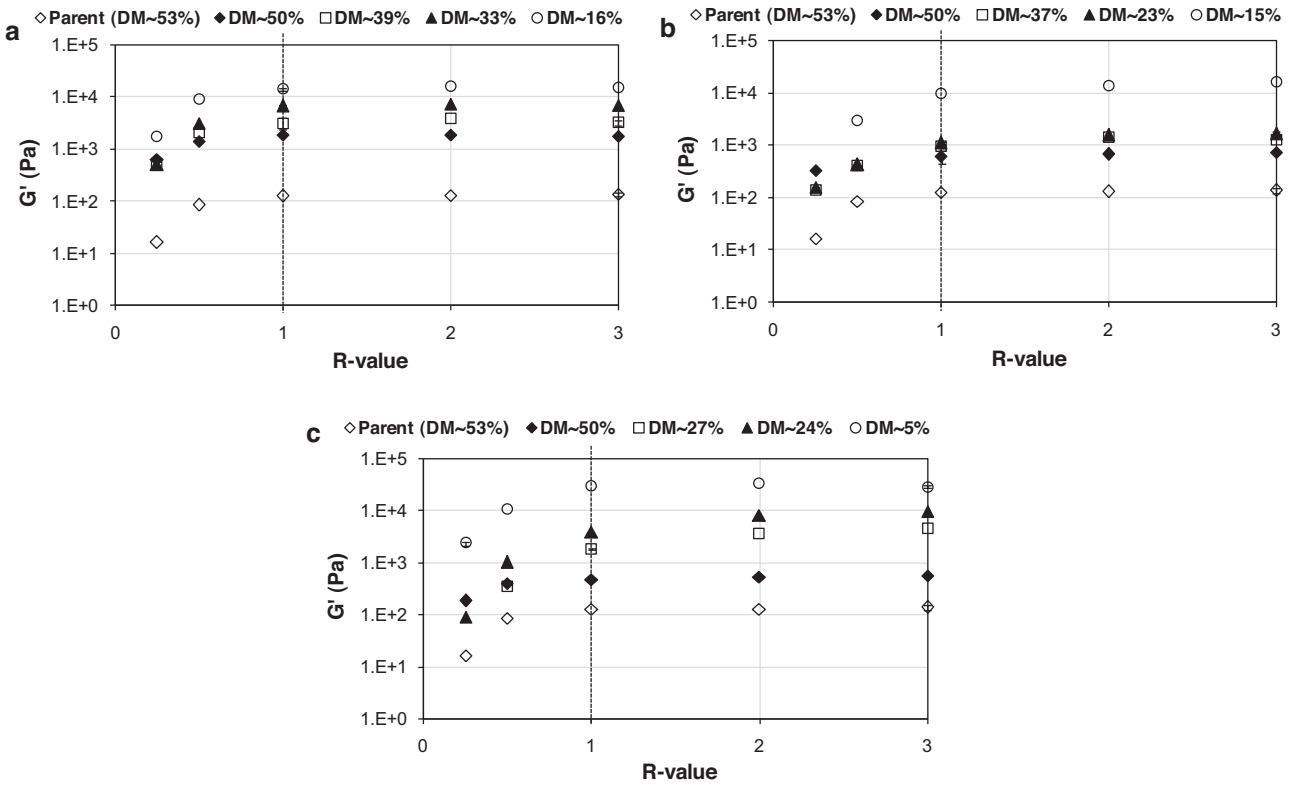

Fig. 5. Gel stiffness (G' at 1 rad/s) of (a) Ca^{2+}-P-mPGA, (b) Ca^{2+}-F-mPGA and (c) Ca^{2+}-C-mPGA gels as a function of Ca^{2+} concentration (expressed as R-value). The dotted line indicates a R-value = 1.0, at which most gels reach the plateau gel stiffness.

upon chemical and fungal PME de-esterification, NM-GalA blocks occurred on many C- and F-mPGAs chains at a time (with C-mPGAs having an homogeneous intermolecular distribution of NM-GalA blocks at all DMs) whereas, upon plant PME de-esterification, additional NM-GalA blocks were sequentially produced on P-mPGA chains (i.e. one chain after the other). Similar intermolecular PM have been reported earlier for pectin de-esterified enzymatically (plant and fungal PME) or chemically (Fraeye et al., 2007; Limberg et al., 2000).

In conclusion, at similar DM, P-, F- and C-mPGAs display a different pattern of methylesterification, despite the relatively low DM (~53%) of the parent mPGA. In a polymer produced through a combination of processes, the final pattern of methylesterification depends not only on the extent of de-esterification but on each modification process.

3.2. Rheological characteristics of Ca^{2+}-mPGA gels

mPGA solutions (pH 6.0) were used for the preparation of Ca^{2+}-mPGA gels at various Ca^{2+} concentrations (expressed as R-value). During gel formation, time sweep tests were carried out to assess the structure development of all gels. After gel formation, the examination of the gels' mechanical spectra (frequency dependence of the moduli and tan δ (ratio of G'' to G')) enabled to evaluate the nature of the mPGA gels (Rao, 1999; Mezger, 2006). The stiffness of the formed gels, determined at constant angular frequency (1 rad/s), was also examined. The structural features of mPGA were used to explain gel properties, thereby allowing to describe structure–function relations.

3.2.1. Structure development and nature of Ca^{2+}-mPGA gels

The structure development of Ca^{2+}-mPGA gels was monitored in terms of the evolution of G' with time. Examples are presented in Fig. 4 for the extreme cases of a high DM (parent mPGA, M53) and a very low DM (i.e. ~5%).

Generally, as shown on the displayed examples, gels immediately revealed rather high G', which displayed limited increase during the cooling step followed by the 5 h isothermal period, except for the low Ca^{2+} (R = 0.25) gels of C-mPGA with DM of ~5% whose G' decreased slightly during the 5 h isothermal period. However, low Ca^{2+} (R = 0.25) gels prepared from C-mPGA (DM ~27%, DB_{abs} ~18%) and F-mPGA (DM ~15%, DB_{abs} ~47%) exhibited a strongly decreasing G' with time (results not shown). This behaviour was observed earlier for the low Ca^{2+} gels of C- and F-pectins with DB_{abs} between 9 and 37%. The decrease is likely related, as suggested by Ngouémazong, Tengweh, et al. (2012), to the initial instability of the formed junction zones. Gels with decreasing G' will not be discussed further.

After gel formation, frequency sweep tests were carried out (results not shown). The dynamic moduli of the Ca^{2+}-mPGA gels displayed a negligible dependence on frequency at all Ca^{2+} concentrations studied and for the different degrees and patterns of methylesterification, thereby revealing a "true" gel nature. In addition, all gels displayed high elastic character (average tan $\delta \sim 0.07$) at all studied Ca^{2+} concentrations. These observations indicate that, irrespective of the Ca^{2+} concentration and the distribution pattern of NM-GalA blocks on the mPGA polymers with DM ≤53%, the gels reveal limited polymer chain mobility and restricted loss of stored energy. This implies that, in C- and F-mPGA (DM ≥ 27% and DM ≥ 33% respectively) gels, the presence of very short junction zones has negligible effects on the nature of the Ca^{2+}-mPGA gels. Therefore, in these systems, the nature and elastic character of the studied Ca^{2+}-mPGA gels mainly depend on the pattern of methylesterification imposed on the final polymer through the methylesterification of the parent mPGA.

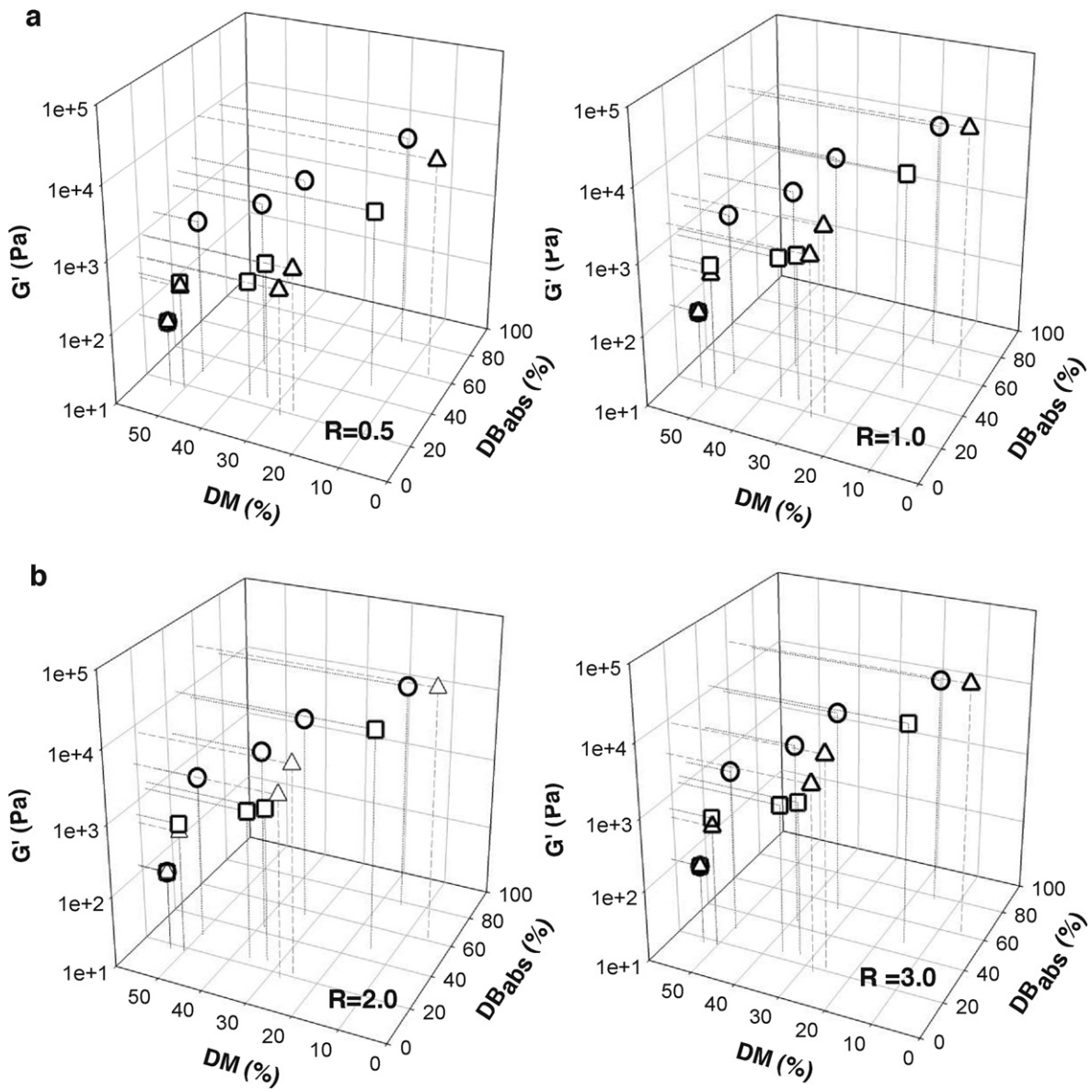

Fig. 6. Combined effects of DM and DB_{abs} on the stiffness (G' at 1 rad/s) of P-mPGA (○), F-mPGA (□) and C-mPGA (△) gels. (a) and (b) represent low and high *R*-values respectively.

3.2.2. *Effect of Ca^{2+} concentration on the gel stiffness of Ca^{2+}-mPGA gels*

The influence of Ca^{2+} concentration on gel stiffness was evaluated for the three types of gels. This enabled to clearly describe how Ca^{2+} concentrations affect junction zones formation in gels prepared from polymers with "mixed" PMs. Comparison of absolute values of the gel stiffness of Ca^{2+}-mPGA gels with those of Ca^{2+}-pectin gels was not possible because, besides the pattern of methylesterification, both polymers differ in many other structural characteristics that influence gel characteristics.

The stiffness (G' at 1 rad/s) of Ca^{2+}-mPGA gels as a function of Ca^{2+} concentration (*R*-value) is depicted in Fig. 5. The stiffness of most Ca^{2+}-mPGA gels increased with *R*-value up to a plateau value which was reached at $R = 1.0$. This observation indicates that, at Ca^{2+} concentrations corresponding to $R = 1.0$, all possible junction zones which can contribute to the stiffness of Ca^{2+}-mPGA gels may be completely formed. Ca^{2+} concentrations at which gels display constant G' are referred to as saturating Ca^{2+} concentrations.

Remarkably, at *R*-values above 1.0, the stiffness of the gels prepared from chemically de-esterified mPGA with DM of about 27% and 24% displayed a continuous slightly increasing trend with increasing *R*-value (Fig. 5c), indicating the formation of additional junction zones in the gels (at higher *R*). A similar gradually increasing trend of G' for *R*-values above 1.0 was observed for gels of F- and C-pectins with high and intermediate DM (Ngouémazong, Tengweh, et al., 2012). A common property of C- and F-pectins of high/intermediate DM and the studied C-mPGA with DM of about 27% and 24% is that they contain a high number of relatively short NM-GalA blocks (with less than five GalA residues) which are separated from one another by stretches of methylesterified GalA residues. As suggested by Ngouémazong, Tengweh, et al. (2012), Ca^{2+} condensation onto these stretches of methyl groups may limit cations availability in the short NM-GalA blocks, particularly at low *R*-values. However, as the Ca^{2+} concentration increased, short NM-GalA blocks of adjacent chains likely became readily cross-linked with Ca^{2+}. Furthermore, the behaviour of these gels suggests that,

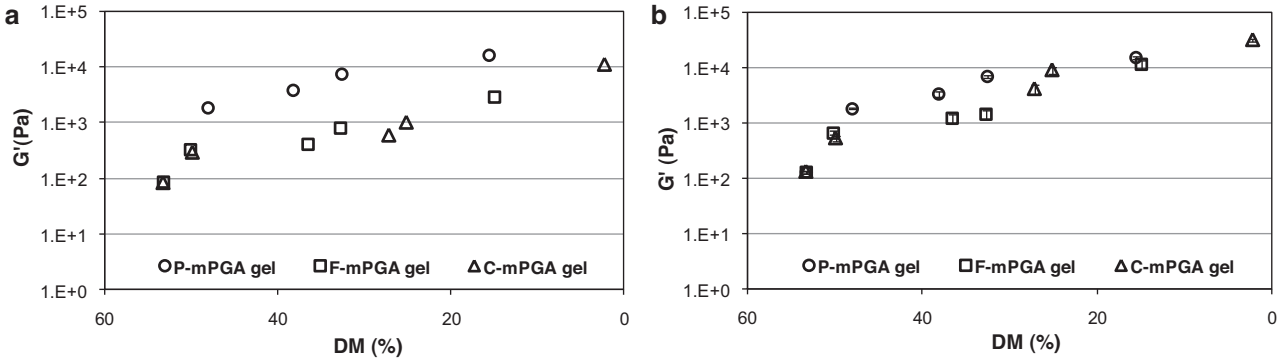

Fig. 7. Effect of DM on the stiffness (G' at 1 rad/s) of P-mPGA (○), F-mPGA (□) and C-mPGA (△) gels. (a) and (b) represent $R = 0.5$ and saturating R-values, respectively.

besides large junction zones, short junction zones also contribute (albeit less) to the stiffness of C-mPGA gels.

3.2.3. Effect of polymer structural features (DM and DB_{abs}) on the gel stiffness of Ca^{2+}-mPGA gels

The combined effects of DM and DB_{abs} on the stiffness (G' at 1 rad/s) of Ca^{2+}-mPGA gels are depicted in Fig. 6 for various Ca^{2+} concentrations (expressed as R-value). Unlike P-mPGA gels, C- and F-mPGA gels displayed similar gel stiffness, both at low and high R-values. Detailed discussion is provided in subsequent sections.

3.2.3.1. Effect of DM on the gel stiffness of Ca^{2+}-mPGA gels. To allow a detailed comparison of the stiffness of P-, F- and C-mPGA gels, a plot of G' as a function of DM for low and saturating R-values is shown in Fig. 7. Remarkably, in all cases, G' increased with decreasing DM, although a decrease in DM is not always accompanied by increase in DB_{abs}, particularly for C- and F-mPGAs. This indicates that the short NM-Gal blocks formed at early stages of chemical and fungal PME de-esterification may positively affect gel stiffness.

At low R-values ($R = 0.5$), P-mPGA gels displayed the highest stiffness, while F- and C-mPGA gels both showed the lowest, when comparing gels prepared from mPGAs with similar DM (Fig. 7a). This observation indicates that, at similar DM, P-mPGA gels have stronger gelling properties as compared to F- and C-mPGA gels. This is similar to the behaviour of P-, F- and C-pectin gels and likely results from the large NM-GalA blocks present on P-mPGAs (Section 3.1) and P-pectins (Ngouémazong et al., 2011). It has been proposed that pectin chains of which the HG domain has large NM-GalA blocks interact with Ca^{2+} through cooperative binding and generate gels with higher G' as compared to those prepared from polymers with shorter NM-GalA blocks (Fraeye et al., 2009; Ström et al., 2007; Willats et al., 2001). The similar gel stiffness of F- and C-mPGA gels at limited Ca^{2+} availability (low R-value) indicates that the variations in the pattern of methylesterification of F- and C-mPGAs do not cause any difference in the stiffness of the resulting gels. This is likely related to the presence of NM-GalA blocks of relatively large size on both types of mPGA, as a result of the partial methylesterification of M53.

At saturating R-values (intermediate and high R-values ($1.0 \leq R \leq 3.0$)), gels prepared from mPGAs with similar DM showed comparable stiffness, except for P-mPGA gels of intermediate DM (i.e. ~40–33%), as depicted in Fig. 7b. These findings are different from previous reports on the gel stiffness of P-, F- and C-pectin gels at high Ca^{2+}-concentrations, which showed comparable values at similar DM (Ngouémazong, Tengweh, et al., 2012). The high stiffness of P-mPGA gels of intermediate DM probably results from the occurrence of NM-GalA blocks on nearly all mPGA chains after chemical esterification so that with plant PME de-esterification (even at early stages), the final pattern of mPGA is characterised by the presence of large NM-GalA blocks on numerous mPGA chains. Usually, in plant PME de-esterified pectins, many chains display large NM-GalA blocks only at later stages of de-esterification (Limberg et al., 2000). Therefore, it is suggested that the high stiffness of P-mPGA gels of intermediate DM results from the intermolecular distribution of NM-GalA blocks on the parent mPGA.

In summary, the gel stiffness of the gels prepared from mPGAs with "mixed" patterns of methylesterification is influenced by the distribution patterns imposed on the polymers by both methylesterification and de-esterification.

3.2.3.2. Effect of DB_{abs} on the gel stiffness of the Ca^{2+} gels prepared from C- and F-mPGAs. In order to elucidate the remarkable increase observed in the gel stiffness of the C- and F-mPGA gels prepared from polymers which revealed constant DB_{abs}, a close look was taken at the effect of DB_{abs} on the gel stiffness (G' at 1 rad/s) of C-mPGA gels, as illustrated in Fig. 8, for gels with low ($R = 0.5$), and saturating Ca^{2+} concentrations ($R = 1.0$ and 3.0). This figure also includes a comparison with P-mPGA gels. F-mPGA gels are omitted as they showed similar behaviour as C-mPGA gels.

At saturating R-values, gels prepared from C-mPGAs of rather similar DB_{abs} (but different DM) displayed a striking increase (by a factor of ~100) in gel stiffness. At low R-value, the difference was less pronounced probably because junction zones were not completely formed. It is worth recalling that C-mPGAs which showed constant DB_{abs} values contain both short NM-GalA blocks with at most four GalA residues as well as large blocks. This suggests that, at saturating Ca^{2+} concentrations, short junction zones consisting of a maximum of four contiguous GalA residues could be formed in the aforementioned C-mPGA gels. So far, it is reported

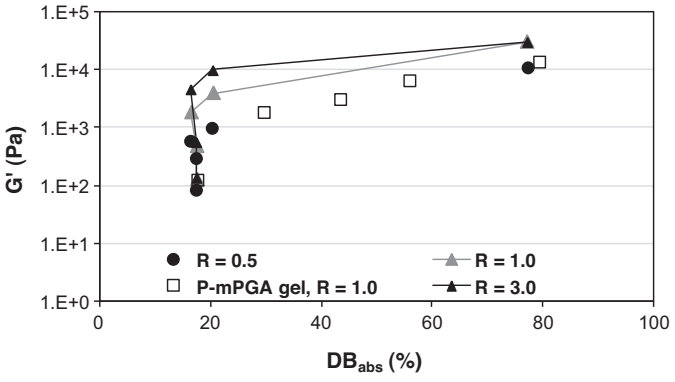

Fig. 8. Gel stiffness (G' at 1 rad/s) as a function of DB_{abs} for C-mPGA gels at low ($R = 0.5$) and saturating ($R = 1.0$ and 3.0) Ca^{2+} concentrations, and P-mPGA gels at $R = 1.0$. The comparison with P-mPGA gels enables to clearly point out the importance of small junction zones in increasing gel stiffness.

that a minimum of six contiguous NM-GalA residues are required for the formation of cooperative junction zones (Luzio & Cameron, 2008). This implies that junction zones with a maximum of four NM-GalA residues are formed through non-cooperative binding. Since gel stiffness increased tremendously as a result of the formation of these short junction zones, we therefore propose that in concentrated pectin solution, non-cooperative junction zones also contribute to the stiffness of Ca^{2+}-mPGA gels. P-mPGA gels, which contain only cooperative junction zones even showed a much less steep increase of gel stiffness with DB_{abs}, at saturating Ca^{2+} concentration.

Finally, it should be noted that a similar steep increase of G' with DB_{abs} was also observed for gels of C- and F-pectins of very low DB_{abs} (Ngouémazong, Tengweh, et al., 2012; Ngouémazong, Kabuye, et al., 2012). Similar to the present case, this was under conditions were small NM-GalA blocks were present on pectin. It was also reported that a high Ca^{2+} concentration was required to be able to generate gel stiffness from the short junction zones.

4. Conclusion

Subjecting poly-D-GalA to partial chemical methylesterification followed by partial de-esterification using plant PME, fungal PME or chemical de-esterification yielded partially methylesterified polymers (mPGA) with various degrees and patterns of methylesterification. The final patterns of methylesterification of the produced mPGAs depend not only on the extent of de-esterification but also on the patterns resulting from the initial "uncontrolled" methylesterification and the controlled de-esterification method. Following the analysis of Ca^{2+} gels prepared from the produced mPGAs, it was observed that the "mixed" patterns of methylesterification greatly affect the rheological characteristics of the gels.

The results of this study reveal the importance of establishing structure–function relations of commercial and *in planta* modified pectins. These findings also suggest that the sequence of the modification processes of pectin's homogalacturonan can be of crucial importance in defining the rheological properties of pectin gels. Consequently, while handling pectins (commercial or *in planta*), either their complete history must be known or the polymer must be thoroughly characterised and a precise structure–function relation specified.

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