Chromatographic and Enzymatic Strategies To Reveal Differences between Amidated Pectins on a Molecular Level

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The intermolecular distributions of amide groups within two commercial LMA pectins was studied after removal of the methyl esters followed by fractionation of the different populations by anion exchange chromatography. The populations obtained had almost equal degrees of amidation while the values of the degree of blockiness were not the same, indicating also a different intramolecular distribution of the substituents considered as semirandom. Populations from the methyl-esterified amidated pectins showed a rather random distribution for almost all populations. A striking difference between these different populations was that, despite the same level of substitution, the ratio between amide groups and methyl esters varied significantly, indicating a heterogeneous amidation process.

Introduction

Nowadays, pectin is widely used as a gelling and thickening compound, but is also known for its health effects such as antidiarrhea and detoxicant properties, the regulation and protection of gastrointestinal tract, and anti-tumor activity.[1,2] It has been demonstrated that pectin in its native state is a complex polysaccharide composed of an α-1,4-linked D-galacturonic acid (GalA) backbone (smooth regions). This homogalacturonan is interrupted by alternating rhamnose/GalA sequences where rhamnose moieties are substituted by neutral sugars (hairy regions).[3−10] Commercial pectins are extracted from citrus peels or apple pommace, mainly yielding high methyl-esterified (HM) pectins, meaning that 50% or more of the galacturonic acids are methyl-esterified. These HM pectins can be de-esterified to produce low methyl-esterified (LM) pectins (less than 50% of the galacturonic acid residues in the backbone are methyl-esterified). These two types of pectins have completely different gelling conditions. The LM pectins are used mainly in the presence of calcium at neutral pH but also under acidic conditions without calcium.[2,11] HM pectins are used at low pH (below 3.5) in the presence of sugar and without calcium addition.[2] The gels of LM pectins are known to be shear reversible, which is not the case for the HM pectin gels.[12] A third category of pectin is obtained by chemical amidation of HM pectins to obtain low methyl-esterified amidated pectins (LMA pectins). These LMA pectins need less calcium to gel and are claimed to be thermoreversible.[13] Furthermore, the firmness and the strength of the gels obtained in the presence of calcium are higher for LMA compared to the LM pectins with a similar degree of substitution.[14]

The gelling mechanism of amidated pectins is not completely understood yet. It seems that both the egg-box mechanism described previously for LM pectins[2] and the stabilization of the junction zones with the hydrogen bonds of amide groups on pectins[15] play an important role. The gelling mechanisms of pectins are influenced by several factors such as their molecular weight,[16−18] their total charge, and the distribution of their charges over the pectic backbone.[2,12,19,20]

In this study, we have characterized in detail, using anion exchange chromatographic separation, two LMA pectins with similar chemical characteristics but different gelling behaviors in the presence of calcium. Pectins and pectic fractions were studied in the original form but also after saponification to study the distribution of amide groups only. Populations were also digested with an *endo*-polygalacturonase to determine the intramolecular distribution of the substituents over the galacturonan backbone.

Material and Methods

Pectin Samples. The samples D and G were kindly provided by Degussa Texturant Systems. The molecular weight as determined by high-performance size exclusion chromatography[26] is respectively 81 and 87 kDa. The galacturonic acid content (GalA), the degree of methyl esterification, and the degree of amidation of these pectins are described in Table 1.

Saponification of Pectins D and G. Pectin samples were wetted with ethanol, solubilized in water (8 g/L), and cooled on ice. Then an equal volume of NaOH (0.1 M) was added. The solutions were stirred and stored overnight at 4 °C. An equal volume of acetic acid (0.1 M) was added to neutralize to pH 7. Acetate and methanol were removed by dialysis with dialysis tubing (cut off 12−14 kDa for proteins) and samples were freeze-dried. No β-elimination occurred as indicated by HPSEC analysis of the saponified pectins (results not shown).

Preparative Chromatography of Commercial Pectins. An Akta explorer system was used for separation of pectins on a preparative scale. Pectin (0.5 g) was dissolved in 100 mL of 0.03 M sodium phosphate buffer (pH 6). Elution was performed on a Source-Q column (115 × 60 mm; Amersham Biosciences) using "Millipore" water for 4 column volumes (CV) followed by a linear gradient in steps: 0 to 0.12 M sodium phosphate buffer (pH 6) in 13 CV at 60 mL/min; 0.12 to 0.42 M sodium phosphate buffer (pH 6) in 44 CV; 0.42 to 0.6 M sodium

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Table 1. Characteristics of Crude and Saponified LMA Pectins D and G as Well as the Populations Obtained after Fractionation on a Preparative Source-Q Column of the Saponified LMA Pectins Dsap and Gsap

samples	recovery[a] (%)	GalA (w/w %)	NS (w/w %)	DAm[b] (%)	DB[c] (%)	DB_{abs} (%)
D		68	5	19 (DM29)	9	4
Dsap		71	5	19	8	6
$D1_s$	2	7	16			
$D2_s$	25	65	3	22	11	9
$D3_s$	46	70	2	20	14	11
$D4_s$	27	69	2	15	23	19
G		70	5	18 (DM31)	9	4
Gsap		69	5	18	9	7
$G1_s$	1	14	5			
$G2a_s$	6	22	4	24	11	9
$G2b_s$	4	35	2	20	15	12
$G2c_s$	10	61	2	17	14	12
$G3_s$	33	68	2	16	9	8
$G4_s$	42	65	2	16	17	14
$G5_s$	4	4	1	19	34	28

[a] Percentage of dry material recovered. [b] DAm determined using CE method. [c] DB determined with HPAEC method.

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

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

Uronic Acid Content. Pectin solutions (60 μg/mL) were boiled (1 h), cooled, and then saponified with sodium hydroxide (40 mM). The uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl method.[21−23]

Neutral Sugar Content. The neutral sugar composition was determined by gas chromatography[24] using inositol as an internal standard. The samples were treated with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C and the constituent sugars released were analyzed as their alditol acetates.

Methyl-Ester Content. The methyl-ester content was determined by GC headspace analysis of the free methanol released after alkaline de-esterification of pectins.[25]

Determination of the Degree of Blockiness by *endo*-pG Digestion of Pectins. Pectins (5 mg/mL) were dissolved in 50 mM sodium acetate (pH 5) and incubated with an overdose of *endo*-polygalacturonase of *Kluyveromyces fragilis* (0.04 units/mL) for 24 h. The specific activity of this enzyme for PGA was 128 units/mg. As a result of the extended *endo*-polygalacturonase incubation employed, only end products were observed, as was demonstrated by the use of higher enzyme doses and longer incubation times. Oligomers released were analyzed by HPAEC or CE as described below and the degree of blockiness was calculated. The degree of blockiness (DB) is the amount of mono-, di-, and trigalacturonic acid released by the *endo*-polygalacturonase related to the amount of free GalA present in the sample. The absolute degree of blockiness (DB_{abs}) is the amount of mono-, di-, and trigalacturonic acid released by the *endo*-polygalacturonase related to the total amount of GalA (free and substituted GalA) present in the sample.[26]

Determination of the Degree of Amidation, Degree of Substitution, and Degree of Blockiness of Amidated Pectins by Using CE. Analysis of the degree of amidation was performed as described previously.[27] Phosphate buffer (50 mM, pH 7) was used as electrophoresis buffer. Samples and standards were wetted in 10 μL of ethanol and dissolved in the phosphate buffer (5 mg/mL). Experiments were carried out on an automated CE system (P/ACE MDQ) equipped with an UV detector (stated at 190 and 200 nm). A fused silica capillary internal diameter of 50 μm and total length of 50.2 cm with 40 cm length capillary from inlet to detector was used and thermostated at 25 °C. New capillaries were conditioned by rinsing for 15 min with 0.1 M NaOH, 30 min with distilled water, and 30 min with phosphate buffer. Between two runs the capillary was washed for 2 min with 0.1 M NaOH, 1 min with distilled water, and 2 min with phosphate buffer. All solutions were filtered on a 0.2 μm membrane. Samples (50 μL) were loaded hydrodynamically (5 s at 9.5 psi) and electrophoresis was performed across a potential difference of 20 kV (for 37 min in phosphate buffer, pH 7) for DS, DM, and DAm analysis and 17 kV for the DB analysis (performed on the populations fractionated from the crude pectins D and G). The separation process is performed in normal polarity.

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

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

$$EM = EM_p - EM_m = (l \times L/V)[(1/t_p) - (1/t_m)]$$

where EM_p corresponds to the observed mobility of the pectin and EM_m to the observed mobility of the eof, l is the distance from the inlet to the detector, L is the total length of the capillary, V is the applied voltage, and t_p and t_m are the migration times of pectins and neutral markers, respectively.[28]

Analysis of Galacturonic Acid Oligomers for Determination of the Degree of Blockiness (by Using HPAEC pH5). Oligomers released in *endo*-polygalacturonase digests (of the populations fractionated from Dsap and Gsap) were analyzed by HPAEC on a ThermoQuest HPLC system (100 μL injection) equipped with a Dionex CarboPac PA1 anion exchange column (250 × 2 mm) and a CarboPac PA1 precolumn (50 × 2 mm). The column was equilibrated with 0.01 M sodium acetate (pH 5) for 10 min. Elution was performed in two steps: a linear gradient from 0.01 to 0.55 M of sodium acetate (pH 5) in 40 min and another linear gradient from 0.55 to 1 M sodium acetate (pH 5) in 60 min with a flow of 0.2 mL/min. The gradient was held at 1 M sodium acetate (pH 5) for 10 min. The PAD detector (Dionex) was equipped with a gold working electrode and an Ag/AgCl reference electrode. Detection of the oligomers was possible after postcolumn sodium hydroxide addition (1 M; 0.2 mL/min). Mono-, di-, and triGalA peaks were integrated by using the peakfit software (Aspire Software International).

Results and Discussion

Separation of Pectic Populations Obtained from Saponified LMA Pectins by Preparative Anion Exchange Chromatography. Two LMA pectins were analyzed to understand their different gelling behaviors in the presence of calcium. Pectin D was found to be more sensitive to calcium compared to pectin G during gel formation (results not shown), but routine chemical analysis (GalA and NS content, DM and DAm; Table 1) showed similar chemical characteristics. The degree of blockiness (DB), which is a parameter to reveal the distribution of the charges over the pectic backbone, has been introduced previously.[26,29,30] To establish the DB, pectins are digested with an *endo*-polygalacturonase known to release mono-, di-, and triGalA oligomers when sequences of more than four free GalA blocks are present. The DB is the percentage of these non-

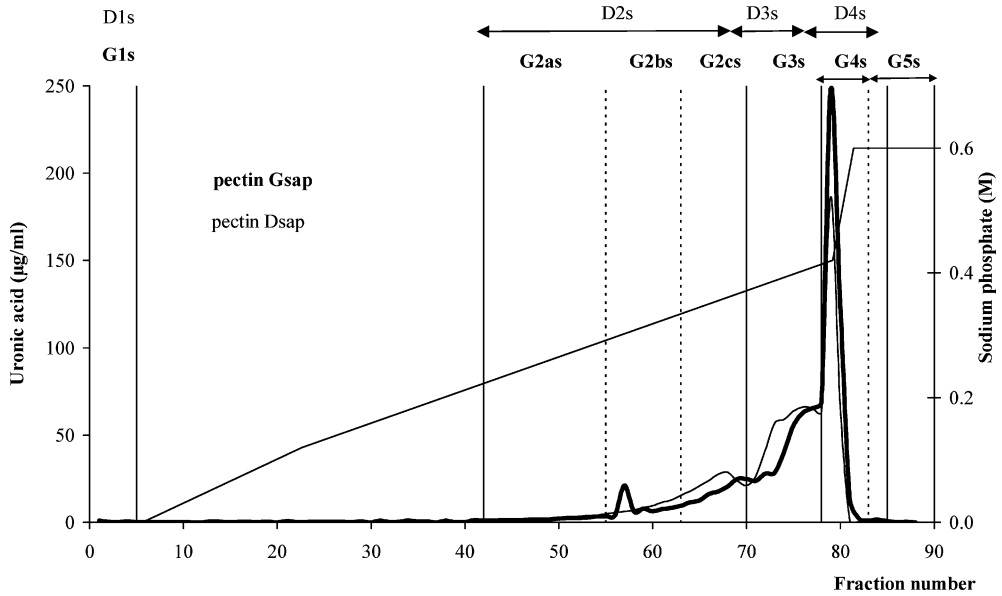

Figure 1. Preparative anion exchange chromatography of saponified pectins Dsap and Gsap on a Source-Q column. The elution profiles were obtained after determination of the uronic acid content in each fraction. The fractions (250 mL) were pooled as indicated.

methyl-esterified GalA oligomers liberated by the *endo*-PG related to the total number of non-methyl-esterified GalA present in the pectin.[26,29,30]

It has been suggested that amide groups and methyl esters had the same effect on *endo*-PG action when pectins were digested.[31,32] With use of the amount of mono-, di-, and triGalA released by the *endo*-PG, the DB of the amidated pectins was determined. Since the DB of pectins D and G was found to be similar (9%), this parameter did not explain their different gelling behaviors.

The distribution of the substituents of these pectins were rather random since Daas et al. found a higher DB for a blockwise methyl-esterified pectin with a DS similar to that of the amidated pectins (33% for a DM 56.4 pectin).[33]

Recently, commercial HM pectin preparations were found to be composed of populations with different characteristics concerning the total charge and the distribution of these charges,[26] which may account for the different gelling behaviors of the pectins. The amidated pectin preparations were suspected to contain different pectin populations with different chemical features as well; therefore, the pectic populations of amidated pectins were separated by preparative anion exchange chromatography by using the same approach as described previously.[26]

Since LMA pectins contain both methyl esters and amide groups, we saponified pectins to focus first on the distribution of the amide groups. The DB of saponified pectin G (Gsap, DB 9%; Table 1) was slightly higher than the one of saponified pectin D (Dsap, DB 8%), indicating a slightly more blockwise distribution of the amide groups in pectin G compared to pectin D. However, these rather low DB values indicated a rather random distribution of the amide groups compared to a blockwise methyl-esterified pectin (DB of 39% for a DM 17 pectin) with similar DS.[33] From the similar DB of the crude amidated pectins compared to the saponified ones, methyl esters were suggested to be regularly distributed in such a way that removal of these esters does not influence the size of the PG degradable blocks.

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

The elution profiles of saponified pectins Dsap and Gsap were rather similar with only differences in the relative proportion of the populations present (fractions 40−69, 70−77, and 78−83, respectively). Pectin Gsap contained slightly more pectin molecules eluting at high ionic strength and less pectin molecules eluting at lower ionic strength compared to pectin Dsap. Neutral sugars were found mainly in population D1s eluting at low ionic strength (16% in w/w; Table 1) and the NS content was low for the other populations from pectins Dsap and Gsap (1 to 5% in w/w).

The populations may differ in their total charge and/or in the distribution of the charges since it has been demonstrated that the elution behavior of pectins on this column is sensitive to these two different features.[26] Fractions were collected and pooled as shown in Figure 1 and characterized (Table 1). The recovery of GalA content was 89% and 91% for pectins Dsap and Gsap, respectively.

The GalA content was low for populations eluting at low ionic strength ($D1_s$, $G1_s$, $G2a_s$, and $G2b_s$) and high ionic strength ($G5_s$) as has been observed previously for methyl-esterified pectins.[34] These populations were not investigated further since they represented less than 5% of the total GalA present in the crude pectin. The NS content was low for both commercial pectins as a result of the acid extraction in the manufacturing process.[26,35]

The other populations had higher GalA contents: 61 to 70% in w/w. The degree of amidation as measured by CE[27] decreased for populations eluted at high ionic strength of the eluting buffer: from 22 to 15% for $D2_s$−$D4_s$ and from 24 to 16% for $G2a_s$−$G4_s$. The pectin fraction $G5_s$ was deviating from this rule since the DAm was slightly higher compared to the DAm of pectin $G4_s$ (19 and 16%, respectively). The area of each population as defined in Figure 1 was integrated by using the software "Peakfit" to calculate the recovery of amide groups. The recovery of amide groups was 100% for Dsap populations and 84% for Gsap populations. The elution behavior of the populations on the anion exchanger could not be explained by the DAm only since populations $G3_s$ and $G4_s$ for example had the same DAm whereas they eluted at different ionic strength. The parameter reflecting the distributions of free and amidated carboxyl groups (degree of blockiness) was determined for the relevant populations. In addition to differences in DAms, $D4_s$ was found to have a more blockwise distribution of the amide groups compared to $D2_s$ and $D3_s$. An increase of PG degradable

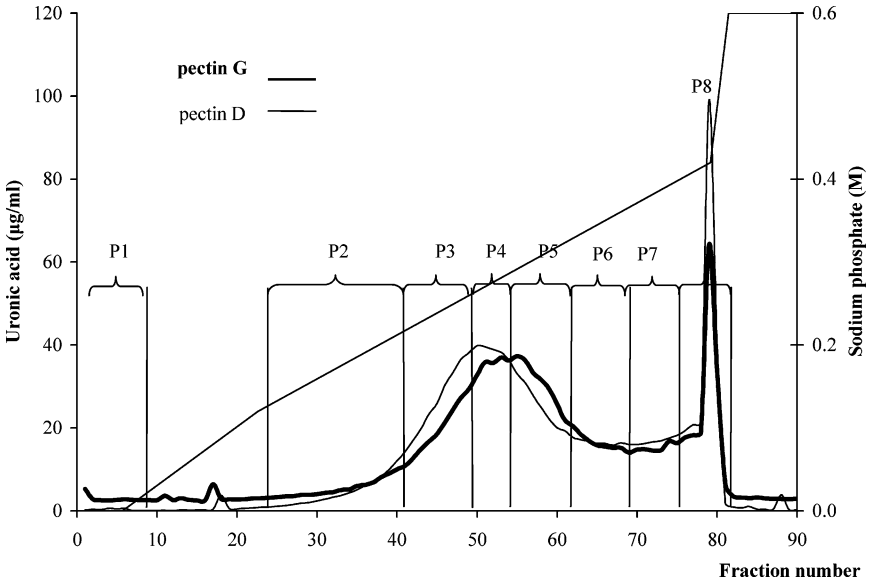

Figure 2. Preparative anion exchange chromatography of pectins D and G on a Source-Q column. The elution profiles were obtained after determination of the uronic acid content in each fraction. The fractions (250 mL) were pooled as indicated.

blocks was also observed for populations $G3_s$, $G4_s$, and $G5_s$ (DB of 9, 17, and 34%, respectively). The populations $G3_s$ and $G4_s$ presented the same DAm but $G4_s$ had a more blockwise distribution of amide groups, explaining the later elution of this population. The late elution time of population $G5_s$ also had to be attributed to a considerably more blockwise distribution of the free GalA (DB of 34%) since the DAm was even higher compared to populations $G2c_s$, $G3_s$, and $G4_s$.

Recently, we also introduced the DB_{abs} corresponding to the ratio of GalA residues released from *endo*-polygalacturonase and the total number of GalA residues (substituted and non-substituted ones) in the pectic population. The Source-Q column was found to discriminate between pectic populations with different DB_{abs}'s.[26] The more *endo*-PG degradable blocks in the pectic populations, the later the elution on the anion exchanger. This was observed as well in this study except for the population $G3_s$.

When we compared the characteristics of Dsap and Gsap populations, we observed that even though populations eluted at the same ionic strength, they differed in their DAm and in the distribution of the amide groups. For example, the populations $D3_s$ and $G3_s$ were found to have a DAm of 20 and 16% and a DB_{abs} of 11 and 8%, respectively.

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

Separation of Pectic Populations from Crude LMA Pectins on Preparative Anion Exchange Chromatography. Pectins D and G were fractionated by preparative anion exchange chromatography (Figure 2). Obviously, pectic populations from pectins D and G eluted earlier compared to those of pectin Dsap and Gsap as a result of the higher degree of substitution (DS of 48 and 49% compared to 19 and 18%, respectively) and consequently lower net to charge. Both crude pectins showed quite similar elution profiles, but pectin D contained more pectin molecules eluting at lowest ionic strength (from 0.2 to 0.25 M phosphate buffer) and at high ionic strength (0.4 M phosphate buffer) compared to pectin G. Fractions were pooled for both pectins D and G as shown in Figure 2 and characterized (Table 2).

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

The NS content was high for the unbound fractions (D1 and G1) and for pectin D2 (50, 24, and 15% in w/w, respectively) and was lower for the other fractions (2−6% in w/w). The degree of substitution was different for the pectic populations: 40−22% for D3−D8 and 41−20% for G2−G8 (w/w). The lower the DS, the greater the binding to the anion exchanger. The DM of the populations was determined by using gas chromatography. The DM was found to decrease when populations were eluted at higher ionic strength (32% for D3 until 3% for D8 and 35% for G3 until 2% for G8). Pectic populations D1 and D2 contained a lower amount of methyl esters and not enough sample was available to perform DS and DAm determinations but these populations D1 and D2 represented only a small part of the dry material recovered (2 to 3%, respectively, Table 2). The DAm was higher when populations eluted at higher ionic strength (8−17% for D3−D8 and 3−18% for G3−G8). Only pectins D4, G2, and G4 were deviating from this rule (respectively, DAm of 6, 18, and 0%).

In general, the more blockwise free GalA are distributed (DB_{abs} from 15 to 42% for D3−D8 and 14−46% for G2−G8), the later the elution of the pectins. The absolute amount of free GalA blocks (DB_{abs}) was influencing the behavior of the pectic populations on the anion exchanger used and the same observation has been reported previously for the elution of populations from HM pectins.[26]

A striking difference in the characteristics of the populations was the proportion of amide groups and methyl esters while the DS of the populations was rather similar. For example, pectins G5 and G6 with similar DS (34 and 33%, respectively) eluted at different ionic strengths. Their Am/Me ratio was different: 0.2 and 0.7, respectively (Table 2). When the ratio of amide groups versus methyl esters (Am/Me) was higher, the elution of the pectin was later. The same phenomenon was observed for other populations with similar DS such as G7−G8 and D3−D5.

We may speculate that amide groups are stabilizing the carboxylate groups, resulting in lower pK_a values for amidated

Table 2. Characteristics of the Populations of LMA Pectins D and G Fractionated on a Preparative Source-Q Column

samples	yield[a] (%)	GalA (w/w %)	NS (w/w %)	DS[b] (%)	DM[c] (%)	DAm[d] (%)	ratio (Am/Me) (%)	DB[e] (%)	DB_{abs} (%)
D		68	5	48 ± 0.5	29	19		9	4
D1	2	4	50	nd	15				
D2	3	48	15	nd	42				
D3	22	73	4	40 ± 0.4	32 ± 0.5	8	0.3	25	15
D4	17	65	5	38 ± 0.7	32 ± 0.5	6	0.3	36	22
D5	15	65	5	39 ± 1.6	25 ± 0.5	14	0.7	44	27
D6	11	62	6	34 ± 0.6	18 ± 0.5	16	1	41	27
D7	14	61	6	24 ± 2.5	9 ± 1.0	15	3	52	40
D8	16	57	4	22 ± 1.3	3 ± 0	17	6.6	54	42
G		70	5	49 ± 0.1	31	18		9	4
G1	3	10	24		2				
G2	11	38	6	41 ± 1	23 ± 0	18	0.8	24	14
G3	15	63	6	38 ± 0.2	35 ± 1.0	3	0.1	35	22
G4	13	56	4	40 ± 0.5	40 ± 0.5	0	0	39	23
G5	13	76	2	34 ± 2.6	29 ± 0.5	5	0.2	44	29
G6	8	57	5	33 ± 0.2	20 ± 0.5	13	0.7	54	33
G7	11	59	5	24 ± 1.9	11 ± 0.5	13	1.2	44	33
G8	26	64	3	20 ± 2.6	2 ± 0	18	9	58	46

[a] Percentage of dry material recovered. [b] DS determined with the CE method. [c] DM determined with the GC method. [d] DAm = DS−DM. [e] DB determined with the CE method.

pectins.[36] This would explain stronger interaction of amidated pectins with the anion exchanger compared to methyl-esterified pectins.

A summary of our observations concerning the different parameters (DS, DM, Dam, and ratio amide groups versus methyl esters) of the pectic populations from pectins D and G is given in Figure 3. For both pectins D and G, the DS of the populations 3 to 6 was found to be rather similar and lower for the populations 7 and 8. The DM was decreasing for pectin D populations eluting later from the anion exchanger while the DAm and the ratio Am/Me were increasing. The same phenomenon was observed for pectin G populations except for the DM, which increased for populations 2 to 4 and then decreased. The DS of populations eluting with the same ionic strength were found to be similar. The higher calcium sensitivity of pectin D compared to pectin G may be attributed to a more blockwise distribution of its substituents in some of its populations (D5, D7, and D8) and to a different ratio amide groups versus methyl esters.

Conclusions

Our study revealed important variations in the chemical features of pectic populations present in commercial amidated pectins. The results showed differences in the degree of substitution (DS from 20 until 41%), although the populations making up the largest part of the commercial pectin did have rather similar levels of substitution (DS ≈ 40%). Pectic populations purified differ also in the distributions of the substituents (DB). However, others parameters were found to fluctuate as well: the ratio between methyl esters and amide groups changed significantly (0.1−9), indicating the presence of pectins almost without methyl esters being present next to molecules rather poor in amide groups. This observation proved the frequently stated suggestion that the amidation process by using a heterogeneous system (insoluble pectins suspended in ethanol) leads to a heterogeneous distribution. However, starting from a HM pectin (DM 70−50), it was surprising that the DS

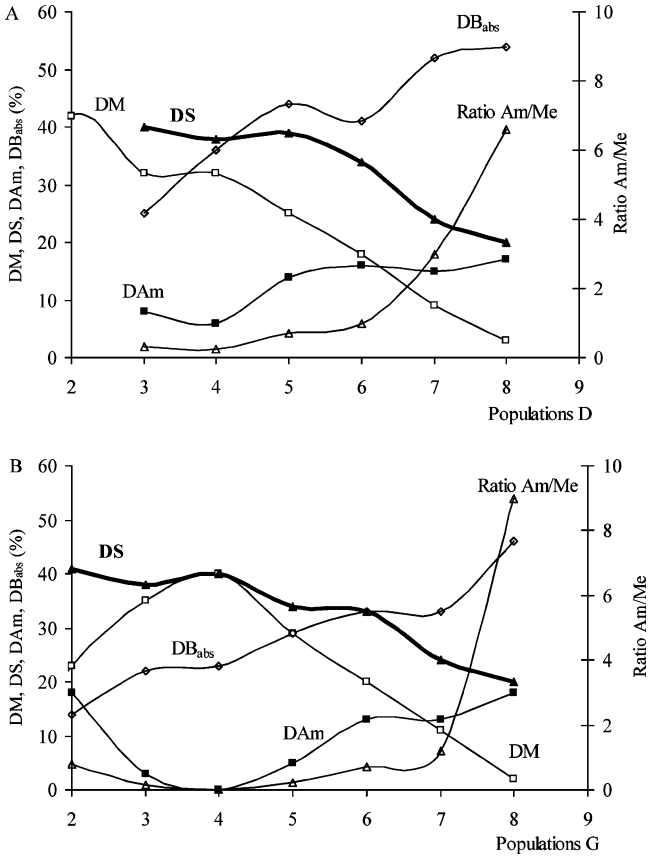

Figure 3. DM, DS, Dam, and DB_{abs} of pectic populations from LMA pectins D (A) and G (B).

was lowered to the same level (ca 40%), despite a different type of substitution. Furthermore, PG degradation studies of LMA pectin fractions before and after removal of the methyl esters resulted in rather similar degradation products and limits. The fact that the amide group distribution rather than the methyl-ester distribution dictate the PG degradability in our set of amidated pectins could be explained by a rather regular methyl-

ester distribution along the molecule, mixed in such a way with the amide groups present that removal of the methyl esters did not create additional sites for *endo*-PG.

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