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Studies on the intermolecular distribution of industrial pectins by means of preparative size exclusion chromatography

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Three industrial high methoxyl pectins have been fractionated by size exclusion chromatography (SEC) on a preparative scale and the chemical composition, viscosity and light scattering behaviour of the fractions have been investigated. Chemical analysis revealed that the composition varies greatly from one SEC fraction to another. In all three pectin samples, the fractions of low molecular size contain most of the free neutral polysaccharides as well as some free pectin 'hairy regions'. In addition, the lemon pectin samples contain some pectin molecules of large size which are rich in neutral sugars. Phenolic and proteinaceous compounds coelute with neutral sugar-rich fractions. However, in the apple pectin, phenolics and proteins occur predominantly in the fractions of low molecular size. Lemon pectin molecules, especially that of the lemon A sample, are prone to aggregation in the presence of calcium cations. The aggregate fraction can be disrupted by shear forces, heating or the presence of a chelating agent. The formation of such calcium–pectinate aggregates seems to be due to the presence of some molecules with low degrees of methoxylation. Light scattering measurements also suggest that even very narrow SEC fractions remain highly heterogeneous on the basis of their molecular weight, thus indicating large differences in molecular conformation.

INTRODUCTION

In a previous paper (Kravtchenko *et al.*, 1992), the chemical composition of three industrial high methoxyl pectins from apple and lemon have been extensively investigated. Although they are known to behave very differently in applications, especially in the presence of calcium ions, the two lemon pectins have been shown to be chemically very similar to each other. In contrast, the apple pectin is richer in neutral sugars, which are present either as neutral side chains or as free neutral polysaccharides, but does not behave very differently from one of the lemon pectin samples in applications. Extensive analysis of whole pectin samples only provided average values and was thus not sufficient to reveal the structural differences that could explain the behaviour in applications.

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With the many possible variations in methoxylation, acetylation, content and type of binding of neutral sugars, content of non-glycosidic residues and molecular size, it is very unlikely that in a given preparation, one pectin molecule is identical to another. High performance size exclusion chromatography (HPSEC) gave information about heterogeneity in the molecular size of pectin molecules within the three pectin preparations investigated (Kravtchenko *et al.*, 1992), but data about the intermolecular distribution of the chemical composition of pectins can only be obtained by extensive fractionation.

Size exclusion chromatography (SEC) is a well known method of separating substances differing in molecular size. In SEC, the elution volume of a molecule of given size depends on its hydrodynamic volume (Yau *et al.*, 1979; Kato *et al.*, 1983). Since the molecular size distribution of a polymer can be important in understanding its functional behaviour (Mitchell, 1976), SEC has often been used to fractionate

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pectin preparations (Jordan & Brant, 1978; Davis *et al.*, 1980; Anger & Berth, 1985, 1986; Rombouts & Thibault, 1986; Hourdet & Muller, 1987; Lecacheux *et al.*, 1987).

In this paper we describe the intermolecular distribution of sugar units, methoxyl and acetyl groups, phenolics, proteinaceous compounds and molecular parameters obtained by viscosity and light scattering measurements for three industrial pectins using preparative SEC.

MATERIALS AND METHODS

Pectin samples

Three unstandardized industrial pectins classified as 'rapid set' were obtained from Sanofi Bio Industries (France): two extracted from lemon peels (lemon A and B) and one from apple pomace. Their chemical composition has been extensively described in a previous paper (Kravtchenko *et al.*, 1992).

Fractionation on Sepharose CL-2B/Sepharose CL-4B

Pectin samples were fractionated on two coupled columns (2·5 × 40 cm), one packed with Sepharose CL-2B and the other packed with Sepharose CL-4B (Pharmacia, Sweden). The fractionation range as determined with dextrans is 3×10^4–5×10^6 daltons for the CL-4B gel and 10^5–2×10^7 daltons for the CL-2B gel. The columns were eluted with 0·037 M phosphate buffer, pH 6·5, containing 0·001 M Na_2-EDTA, and the polysaccharide concentration was recorded continuously with a differential refractometer (Knauer, Germany). For each SEC run, 30 mg pectin were injected. Ten-millilitre fractions were collected for subsequent viscosity and light scattering measurements.

Fractionation on Fractogel TSK HW 55(S)/75(S)

Pectin samples were also fractionated on a larger scale on a column (5 × 90 cm) packed with a mixed bed of Fractogel TSK HW 55(S)/75(S) (1 : 1) (Merck, Germany). The fractionation range as determined with dextrans is 500–2×10^5 daltons for the 55(S) gel and 10^5–10^7 daltons for the 75(S) gel. The column was eluted with 0·1 M Na-succinate buffer, pH 4·8, at a flow rate of 120 ml/h. For each run, 150 mg pectin were injected. Twenty-millilitre fractions were collected, assayed for their uronide and total neutral sugar contents and combined into nine pools. Each pool was ultrafiltered using a PM10 membrane (Amicon, USA) and freeze-dried before further chemical analysis. Corresponding pools from several injections were combined in order to obtain enough material.

Chemical analysis

The anhydrouronic acid (AUA, MW = 176) content was determined by the automated *m*-hydroxydiphenyl assay (*m*hdp, Thibault, 1979). Total neutral sugars were estimated with the automated orcinol assay (Tollier & Robin, 1979), using anhydroarabinose (MW = 132) as a standard.

Neutral sugars were determined by GLC as their alditol acetates (Kravtchenko *et al.*, 1992).

The methoxyl and acetyl contents were determined by HPLC analysis of the methanol and the acetic acid released on alkaline deesterification (Voragen *et al.*, 1986). About 5 mg pectin was saponified with 250 µl of a 0·8 M NaOH/isopropanol (1 : 1) mixture. After centrifugation, 20 µl of the supernatant was injected onto an Aminex HPX87H column for methanol and acetic acid determination. The pectic acid precipitate was redissolved in 25 ml of 0·05 M NH_3-oxalate and the AUA content was determined with the *m*hdp assay.

Protein content was evaluated by the Sedmak & Grossberg assay (1977) using micro-titer plates as described by Rylatt & Parish (1982).

Total phenols were estimated with the Folin-Ciocalteu's reagent without copper treatment using ferulic acid as a standard (Swain & Hillis, 1959) — 0·2 ml of Folin-Ciocalteu's reagent (Merck, Germany) was added to the sample solution (0·4 ml, 0·5%), followed after 5 min by 0·2 ml of saturated Na-carbonate solution. The absorbance at 750 nm was read after 1 h.

High performance size exclusion chromatography

High performance size exclusion chromatography (HPSEC) was performed using a series of Biogel TSK columns (Biorad, USA) 60XL, 40XL and 30XL (300 × 7·5 mm) in combination with a Biogel TSK guard column (75 × 7·5 mm). Columns were eluted with 0·4 M Na-acetate buffer, pH 3·0, at a flow rate of 0·8 ml/min and at 30°C. Detection was performed with a Shodex SE 61 refractive index detector at 40°C.

High performance ion exchange chromatography

High performance ion exchange chromatography (HPIEC) was performed as described by Schols *et al.* (1989). A Biorad MA7P column (50 × 7·8 mm) was eluted with a linear gradient of 15–270 mM Na-phosphate buffer at pH 6·0 with a flow rate of 1·5 ml/min. Detection was carried out by reading the absorbance in UV at 215 nm. The increase in baseline signal was corrected by subtracting the chromatogram obtained for a blank run from those of sample runs.

Physical measurements

Relative viscosities were recorded with a capillary viscoimeter (Viscomatic, Fica, France). Intrinsic viscosities were determined from one single point (Kravtchenko & Pilnik, 1990).

Light scattering measurements were performed with a Sofica light scattering photometer (Fica, France) equipped with a helium-neon laser (Zeiss, Germany) of wavelength 632 nm. Details are given elsewhere (Berth *et al.*, 1990).

RESULTS AND DISCUSSION

Fractionation by preparative SEC

Industrial samples have been fractionated by preparative SEC without any previous purification. Prior to injection, the samples were dissolved in the appropriate buffer by shaking overnight at room temperature. Figures 1 and 2 show the elution patterns obtained by chromatography on Fractogel HW 55(S)/75(S) and Sepharose CL-2B/Sepharose CL-4B, respectively. Recoveries were not significantly different from 100%. Rechromatography on HPSEC as shown in Fig. 3 demonstrates the efficiency of the fractionation on the Fractogel TSK column.

Since pectin molecules from different preparations with the same molecular weight can have different hydrodynamic volumes due to differences in degree of methoxylation (Smidsrød & Haug, 1971; Michel *et al.*, 1982; Fishman *et al.*, 1984) or degree of branching with neutral sugar side chains (Berth, 1988; Kravtchenko *et al.*, 1992), SEC is not suitable for the direct determination of the molecular weight of pectins (Masuda *et al.*, 1979; Anger & Berth, 1985, 1986; Berth, 1988; Kravtchenko *et al.*, 1992). However, simple qualitative inspection of chromatograms may reveal important information about the molecular size distribution of pectin preparations.

Figures 1 and 2 show that the three industrial pectins have large hydrodynamic volumes and wide size distributions. The apple pectin, which elutes somewhat earlier, appears to have a higher average molecular size than the two lemon pectins. However, the apple pectin also contains a higher proportion of smaller molecules which elute in the tail of the main peak, thus resulting in a broader size distribution. These results confirm those obtained previously by low pressure SEC (de Vries *et al.*, 1984; Brigand *et al.*, 1990) and HPSEC (Brigand *et al.*, 1990; Kravtchenko *et al.*, 1992).

Compared with fractionation on the Fractogel TSK column (Fig. 1) or HPSEC (Kravtchenko *et al.*, 1992), the chromatograms obtained with the Sepharose columns (Fig. 2) exhibit a wide distribution for the largest molecules. This may be due to the wide overlap

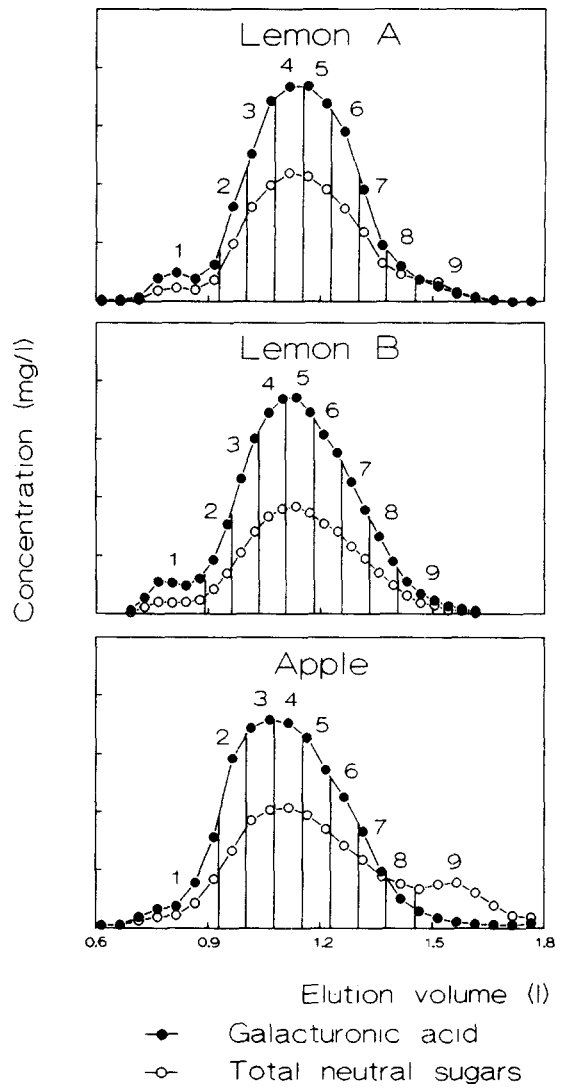

Fig. 1. Elution patterns of the three industrial pectins on Fractogel TSK HW 55(S)/75(S) eluted with 0·1 M Na-succinate buffer at pH 4·8 containing 29 mg/litre of calcium cations.

of the fractionation range for the Sepharose CL-4B and Sepharose CL-2B gels, leading to an increased resolution in that region.

On fractionation on the Fractogel TSK column (Fig. 1) both lemon pectins give a separate peak eluting at the void volume of the column. This early peak disappears when samples are heated at 100°C for about 10 min just before the injection, although the rest of the chromatogram remains unchanged. Moreover, when heated samples are kept at room temperature for a few hours before injection, the peak reappears with the same size. Such a peak does not occur on chromatography on HPSEC (Kravtchenko *et al.*, 1992). On rechromatography on HPSEC (Fig. 3) Fraction 1, especially that of the lemon A sample, exhibits an unexpectedly wide molecular size distribution compared to the other fractions. Indeed the elution pattern of the void fraction obtained from the original separation resembles

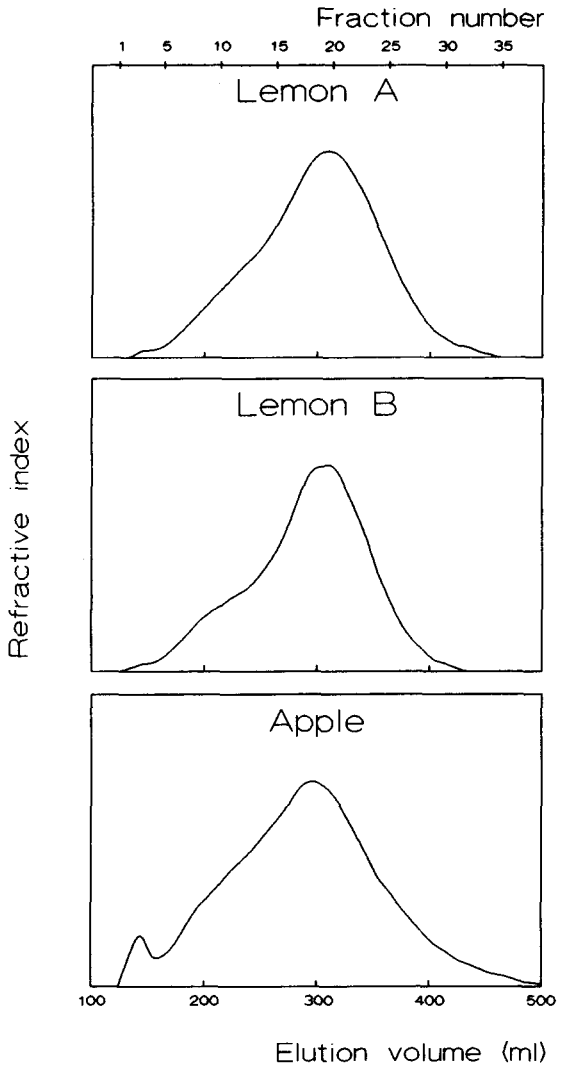

Fig. 2. Elution patterns of the three industrial pectins on Sepharose CL-2B/Sepharose CL-6B eluted with 0·037 M phosphate buffer at pH 6·5 with 0·001 M Na_2-EDTA.

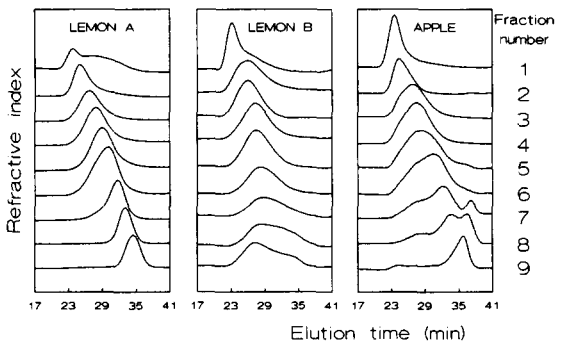

Fig. 3. HPSEC elution patterns of the fractions obtained by preparative SEC on Fractogel HW 55(S)/75(S) for the three industrial pectins as shown in Fig. 1.

that of the corresponding unfractionated pectin sample (Kravtchenko *et al.*, 1992). Discrepancies in elution behaviour might be explained by molecular disruption caused by high shear forces which occur in the HPSEC system. These results are consistent with the hypothesis of the presence of a microgel component in pectin solutions (Sorochan *et al.*, 1971; Smith, 1976; Berth *et al.*, 1977, 1982; Kawabata & Sawayama, 1977; Jordan & Brant, 1978; Davis *et al.*, 1980; Plashchina *et al.*, 1985) which can be disrupted by heat (Sorochan *et al.*, 1971; Smith, 1976; Fishman *et al.*, 1986) or shear forces (Fishman, *et al.*, 1986). For large-scale fractionation on the Fractogel column, the eluant was prepared with tap-water containing 29 mg/litre of calcium. When the buffer was prepared with distilled water, the same voiding peak did not occur. This might indicate the involvement of calcium ions in the phenomenon of aggregation that we have observed.

Comparing chromatograms obtained for samples heated and unheated, this aggregate fraction can be estimated to represent 4 and 2% for lemon A and lemon B, respectively. However, even operating at a relatively low flow rate, the SEC column may still generate shear forces able to disrupt some aggregates. Thus, SEC cannot be considered as a reliable technique to quantify the phenomenon of aggregation.

A similar void peak occurs in the apple pectin sample too, but it proved to remain stable when the sample was preheated prior to injection onto SEC. Moreover, this fraction remains homogeneous on HPSEC (Fig. 3). This indicates that the high molecular size fraction of apple pectin is of a different nature to that of the lemon pectins. It may consist of individual molecules of very large size.

On fractionation on Sepharose columns, the lemon pectins do not show this high molecular size fraction. This might be due to the presence of EDTA in the eluent and may be a further indication of the involvement of divalent cations in the aggregation observed previously on the Fractogel column. On the other hand, the apple pectin sample still exhibits a well separated void peak on Sepharose columns. This seems to confirm our previous interpretation (see above).

Industrial pectin samples have been fractionated on the Fractogel TSK column in order to investigate their intermolecular chemical differences. Since the final goal is a better understanding of the physical behaviour of these pectins, we have decided to use buffer prepared with tap-water and to avoid preheating of samples in order to isolate and further investigate the high molecular size fractions which occur in the lemon pectin samples.

Distribution of neutral sugars

The orcinol assay indicates that the neutral sugar (NS) content is not constant over the pectin molecules fractionated on the Fractogel TSK column (Fig. 1). Results of the orcinol assay have not been corrected for interference with galacturonide. Indeed, we have found

(not published) that the response of galacturonic acid with orcinol depends on its degree of polymerization: polygalacturonic acid gives less colour than monogalacturonic acid, probably because of incomplete hydrolysis during performance of the test. A calculation based on the colour generated by monogalacturonic acid (used as standard) thus underestimates the total NS content. Moreover, the orcinol reagent does not react with the same intensity for different sugar species, and differences in quantity are partly due to differences in sugar composition.

In addition, NSs were also individually determined by GLC in the pools obtained by fractionation on Fractogel TSK. Table 1 gives the NS composition of the fractions as indicated in Fig. 1, expressed as weight % of NS per galacturonic acid.

In lemon pectins, a minimum NS content was found in fractions of intermediate size which represent the main bulk of the samples. Except for Fraction 1, the fractions of high molecular size exhibit a higher amount of NS (about twice that of the molecules of intermediate size), but the most spectacular difference occurs for the smallest molecules. In lemon A pectin, Fraction 9 contains 4 times more rhamnose, 7 times

Table 1. Chemical composition of the fractions obtained by SEC on TSK HW 55(S)/75(S) column (expressed as weight % of AUA)

				Lemon A					
Fraction	1	2	3	4	5	6	7	8	9
AUA (%)[a]	5·9	8·4	16·0	19·6	19·5	15·5	8·2	3·6	2·7
Rha	1·9	2·0	1·7	1·3	1·0	1·3	2·2	2·5	4·3
Fuc	0·4	0·3	0·3	0·3	0·3	0·4	0·4	0·6	1·0
Ara	1·8	2·0	1·5	1·3	1·4	1·7	2·7	4·8	14·7
Xyl	0·3	0·3	0·3	0·2	0·2	0·1	0·2	0·3	1·5
Man	0·4	0·1	0·3	0·3	0·2	0·1	0·2	0·9	4·9
Gal	5·5	5·3	3·7	3·5	3·5	4·6	6·2	17·0	44·4
Glc	0·5	0·3	0·4	0·3	0·3	0·4	0·8	3·0	4·9
Total NS	10·8	10·4	8·1	7·3	6·8	8·6	12·6	28·9	75·6
DM (%)[b]	62·0	70·0	71·0	73·0	72·0	72·0	65·0	66·0	[n.d.]
DAc (%)[b]	1·8	1·9	1·8	1·4	1·4	1·3	1·5	1·9	[n.d.]
Phenolics	0·2	0·1	0·1	0·1	0·1	0·1	0·1	0·6	2·8
Proteins	1·2	0·8	0·7	0·7	0·5	0·5	0·5	0·7	3·8
				Lemon B					
Fraction	1	2	3	4	5	6	7	8	9
AUA (%)[a]	5·7	7·5	13·0	17·4	17·9	15·4	12·0	7·1	4·0
Rha	1·1	1·9	1·5	1·3	1·2	1·2	1·3	1·1	1·5
Fuc	0·2	0·3	0·4	0·2	0·3	0·3	0·3	0·4	0·4
Ara	1·5	3·3	2·0	1·4	1·5	1·7	2·3	2·3	3·0
Xyl	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2
Man	0·2	0·8	0·2	0·1	0·1	0·2	0·7	0·6	0·4
Gal	4·2	5·3	5·4	4·3	4·4	5·4	8·1	8·7	9·4
Glc	0·9	0·5	0·3	0·3	0·5	0·6	1·0	1·2	1·7
Total NS	8·3	12·3	9·9	7·7	8·1	9·6	13·8	14·3	16·6
DM (%)[b]	69·0	75·0	77·0	78·0	73·0	73·0	66·0	67·0	80·0
DAc (%)[b]	1·4	1·9	1·5	1·7	1·3	1·5	1·7	2·3	1·7
Phenolics	0·3	0·4	0·2	0·1	0·1	0·2	0·5	0·6	0·2
Proteins	2·3	1·2	0·7	0·5	0·4	0·6	0·8	0·9	0·5
				Apple					
Fraction	1	2	3	4	5	6	7	8	9
AUA (%)[a]	9·8	14·3	18·3	18·1	15·5	11·5	6·7	2·9	2·0
Rha	1·4	1·7	1·6	1·6	1·4	1·9	2·4	2·2	7·1
Fuc	0·2	0·3	0·2	0·2	0·2	0·3	0·6	0·7	0·4
Ara	2·1	2·1	2·3	2·5	2·8	3·7	7·3	24·5	44·2
Xyl	1·2	1·3	1·3	1·3	1·2	1·3	1·4	1·8	3·0
Man	0·1	0·2	0·1	0·1	0·2	0·3	0·7	1·6	1·6
Gal	3·3	2·9	3·7	3·9	4·4	6·4	11·0	22·5	27·0
Glc	3·4	3·6	7·2	10·8	14·1	16·9	24·5	30·7	52·9
Total NS	11·6	12·2	16·4	20·5	24·2	30·8	48·0	84·0	136·1
DM (%)[b]	76·0	76·0	79·0	76·0	74·0	68·0	56·0	43·0	157·0
DAc (%)[b]	3·6	3·4	4·2	5·2	6·5	5·7	6·8	24·1	[n.d.]
Phenolics	0·8	0·2	0·2	0·1	0·2	0·2	0·6	2·2	2·4
Proteins	1·0	0·6	0·4	0·3	0·5	0·5	1·0	2·2	3·0

[a]Fraction % of the whole sample.
[b]Expressed as mol % of AUA.

more xylose, 10 times more arabinose and 13 times more galactose residues than Fraction 5. This high NS content is partly due to the presence of free neutral polysaccharides which have proved to elute very late on SEC (Le Quéré *et al.*, 1981; Michel *et al.*, 1981; Brigand *et al.*, 1990; Kravtchenko *et al.*, 1992). However, the high content of rhamnose indicates the presence of 'hairy regions' (de Vries *et al.*, 1982).

The high NS content of the largest pectin molecules cannot be explained by the presence of free 'hairy regions' which are parts of the whole molecules. Moreover, since free neutral polysaccharides elute later, it seems that some highly branched pectin molecules of large size occur in lemon pectins. Lecacheux *et al.* (1987), Berth *et al.* (1990) and Berth & Lexow (1991) observed a similar enrichment of NS at the beginning and at the end of the elution pattern of other industrial pectins.

Fraction 1 of the lemon pectin samples exhibits a sugar composition very similar to that of the unfractionated samples, further suggesting that they may be constituted of aggregated molecules of intermediate size.

In the apple pectin, NS content also increases with decreasing molecular size, but the NS content of the largest molecules is not significantly higher than that of the unfractionated sample. The NS content increases regularly from Fraction 5 to Fraction 9.

For all three pectins glucose units which mainly belong to starch molecules (Kravtchenko *et al.*, 1992) are located in the latest fractions, especially in the apple sample. However, a small but significant amount of glucose is present in all fractions, possibly attached to pectin molecules.

Degree of esterification and ester distribution

Table 1 shows degrees of methoxylation (DM) of the fractions obtained by SEC on the Fractogel column from the three industrial pectin samples. DM values measured on very small samples suffered from rather high standard deviations (about 5%). However, we can observe a trend of decreasing DM with decreasing molecular size. Such a variation has already been reported by Brigand *et al.* (1990). In the case of the lemon pectins, especially for lemon A, Fraction 1 exhibits a lower DM than the following fractions. This might explain the tendency of these fractions to aggregate in the presence of calcium cations. On the other hand, Fraction 9 seems to be very highly methoxylated. This is explained by the presence in these fractions of free pectin 'hairy regions' which have been shown to be almost completely methoxylated (de Vries *et al.*, 1982).

Figure 4 shows the elution patterns on high performance ion exchange chromatography (HPIEC) of the SEC fractions from the three pectin samples. Pectin

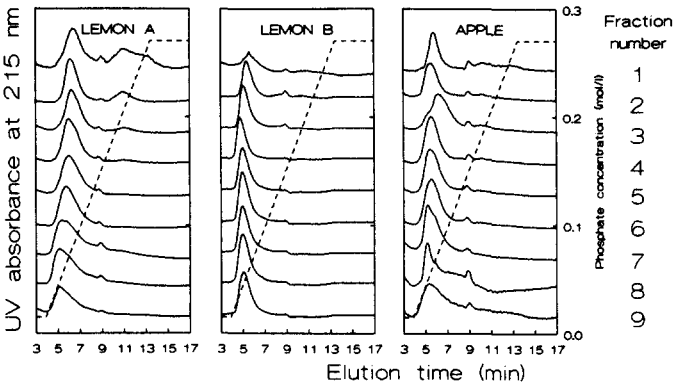

Fig. 4. HPIEC elution patterns of the fractions obtained by preparative SEC on Fractogel HW 55(S)/75(S) for the three industrial pectins as shown in Fig. 1.

fractions clearly elute earlier and earlier with increasing fraction number, i.e. decreasing hydrodynamic volume. As elution volume on IEC is thought to increase with decreasing DM (van Deventer-Schriemer & Pilnik, 1976; Schols *et al.*, 1989) these results seem to contradict those presented above. However, mechanically degraded (Anger *et al.*, 1977) and enzyme degraded (van Deventer-Schriemer & Pilnik, 1987) pectins have been shown to elute at lower ionic strength than undegraded pectins on IEC, making them appear to be more esterified. Differences in elution time on HPIEC may thus be due to some molecular size effect rather than any real difference in DM.

It has been shown (Kravtchenko *et al.*, 1992) that the lemon A pectin sample contains a high proportion of pectin molecules eluting at high ionic strength on HPIEC. Figure 4 shows that almost all these low DM molecules are concentrated in the fractions of high molecular size (Fractions 1-3). Since they do not contain so much of these low DM molecules, lemon B and apple pectin samples do not exhibit such a phenomenon so clearly. The presence of these low DM pectin molecules in the lemon A sample explains the lower average DM found for Fraction 1 and might explain its strong tendency to form aggregates in the presence of calcium (Fishman *et al.*, 1984; Paoletti *et al.*, 1986).

Degree of acetylation (DAc) has also been determined on the fractions obtained by preparative SEC (Table 1). Again with these small samples, the HPLC technique was not accurate enough to provide very reliable results (standard deviations of about 5%). However, it can be seen from Table 1 that the neutral sugar-rich fractions are the most acetylated. This is in agreement with Schols *et al.* (1990) who found a DAc of 60% in neutral sugar-rich pectin fragments from apple. Moreover, it has been shown that acetyl residues are mainly linked to the galacturonosyl residues instead of neutral sugar side chains (Schols *et al.*, 1990). All fractions from the apple pectin sample are more acetylated than those from the lemon samples. This is in agreement with the

fact that apple pectin is more acetylated than lemon pectins (Kravtchenko *et al.*, 1992).

Distribution of phenolic and proteinaceous compounds

Table 1 shows the distribution of total phenolics and proteins over the fractions obtained by fractionation on the TSK column. Phenolics and proteins are present in all fractions from the three pectin samples but in varying amounts. The content of phenolic compounds is the highest in the fractions rich in neutral sugars. This also indicates that they might be associated with pectin molecules via the neutral sugar side chains (Kravtchenko *et al.*, 1992), as is the case for phenolic acids in sugar beet pectin (Rombouts & Thibault, 1986). However, the high molecular size fraction of the apple pectin also contains a high amount of phenolics, although the neutral sugar content is rather low compared to the fractions of lower molecular size. This suggests the presence of some free polyphenols of very large size. Proteins also seem to coelute with the neutral sugar-rich pectin fractions, i.e. fractions of low molecular size. However, the ratio of protein to total neutral sugars decreases regularly with increasing elution volume, suggesting that proteins occur preferentially with the largest pectin molecules. We did not investigate the possible linkage of proteins and phenolics with pectin molecules. Their order of elution on SEC may be either due to their own molecular size or to the size of the pectin molecule to which they are linked.

Viscosity measurements

Relative viscosities of the fractions obtained by fractionation on Sepharose columns have been recorded. Figure 5 shows the intrinsic viscosities plotted on a logarithmic scale against the elution volume. Intrinsic viscosities were calculated on the basis of the concentrations measured by refractometry. As observed repeatedly for other pectin samples (Anger & Berth, 1985, 1986; Berth *et al.*, 1990; Berth & Lexow, 1991) intrinsic viscosity does not increase regularly with hydrodynamic volume. This may be ascribed to the presence of molecules which are built up in different ways. Above an elution volume of about 180 ml, intrinsic viscosity decreases with increasing elution volume. However, the relation is not linear as expected for homologous polymers. The intrinsic viscosity is markedly lowered for both the largest and smallest molecules which were shown to be richer in NS and therefore probably more branched. Surprisingly, the intrinsic viscosity drops rapidly at low elution volumes. This indicates the presence of highly dense and spherical particles which might be either branched pectin molecules or relatively stable aggregates.

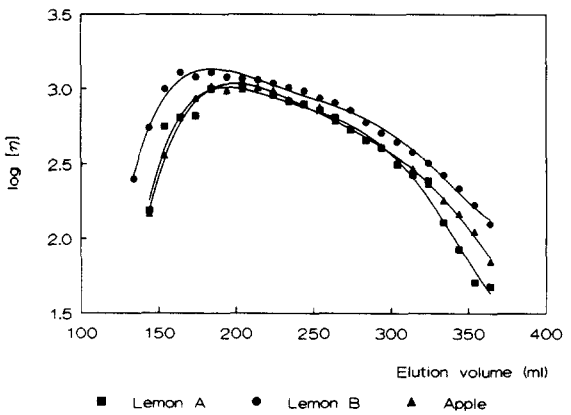

Fig. 5. Intrinsic viscosities of the fractions obtained by preparative SEC on Sepharose CL-2B/Sepharose CL-6B as shown in Fig. 2.

For all fractions, at a given elution volume, lemon B exhibits higher intrinsic viscosities than lemon A and apple pectins. In the light of all the previous observations, the lower intrinsic viscosity of the apple pectin fractions might be due to a higher degree of branching with side chains than the lemon pectins. For the lemon A pectin, the difference might be explained by the presence of some dense particles in all the fractions which do not contribute substantially to the viscosity.

Light scattering measurements

The SEC fractions obtained with the Sepharose columns for the three industrial pectins (Fig. 2) have been investigated by light scattering at angles between 30 and 150°. Measurements were carried out at one single concentration on the solutions resulting from SEC. Since pectin solutions were injected without any preliminary purification and collected in a non dust-free environment, SEC fractions have been filtered prior to light scattering measurements. Results obtained using membrane filters of pore size 0·8 and 0·45 μm are shown as Guinier plots (Kerker, 1969; Berth *et al.*, 1990) in Figs 6 and 7. It should be noted that these data have not been obtained by the analysis of the same fractions after successive filtrations through membrane of diminished pore size, but by the analysis of the fractions obtained from two independent SEC runs. However, SEC fractionation showed excellent reproducibility.

As expected, the scattering intensity decreases with increasing SEC elution volume, indicating decreasing average molecular weights. Only in the case of apple pectin do we observe a slight increase in intensity for the fractions eluting last. That may be explained by the occurrence of compact molecules such as pectin hairy regions and free neutral polysaccharides in the low molecular size fractions (see above).

After filtration through 0·8 μm pore size filters, all the curves exhibit very similar shapes. The strong curvation at angles below 50° followed by a steady flattening above 50° indicates the presence of particles

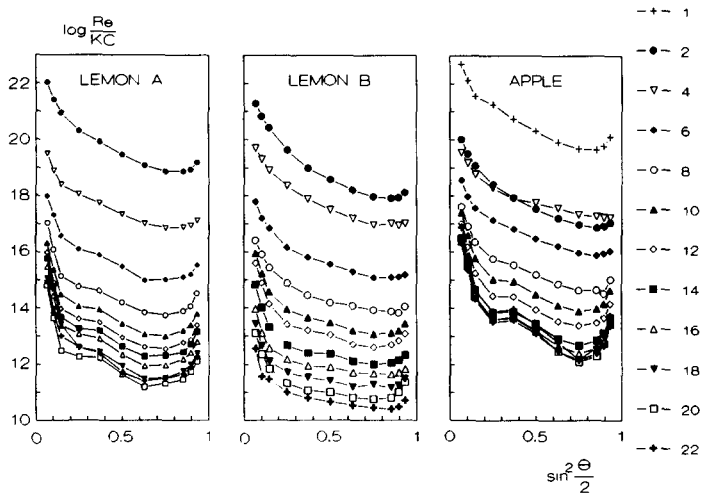

Fig. 6. Guinier plot of the fractions obtained by preparative SEC on Sepharose CL-2B/Sepharose CL-6B as shown in Fig. 2. Fractions were filtrated through 0·8 μm pore size filter prior to light scattering measurements.

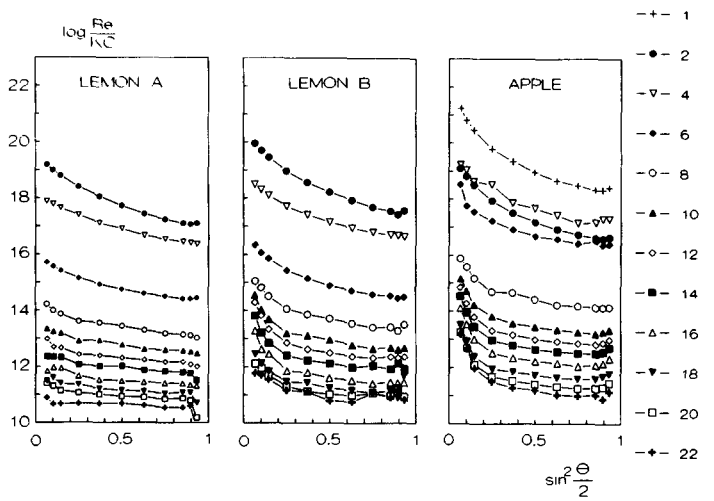

Fig. 7. Guinier plot of the fractions obtained by preparative SEC on Sepharose CL-2B/Sepharose CL-6B for the three industrial pectins as shown in Fig. 2. Fractions were filtrated through 0·45 μm pore size filter prior to light scattering measurements.

of very high molecular weight (Huglin, 1972; Berth *et al.*, 1990). Strongly curved scattering functions indicate an extremely broad mass distribution or bimodal system (Dautzenberg & Rother, 1988). Such an excess of light scattering along the chromatogram has already been reported by Brigand *et al.* (1990). Berth (1988) and Berth *et al.* (1990) also reported the presence of high molecular weight components in SEC fractions of several pectins and pointed out that the very similar curvature within the low angle range suggests a similar particle component of the same size level in all fractions. The upward trends at angles above 135° are probably due to reflections within the measurement system and should be ignored.

The use of 0·45 μm pore size filters instead of 0·8 μm ones reduces considerably the scattering level, although any measurable loss of polymer cannot be detected. Also, scattering curves become more flat and exhibit a more constant angular dependence of the scattered light, indicating the removal of at least some large sphere-like particles. Although dissolved macromolecules can usually pass unrestricted through membranes of the pore sizes applied here, it is clear that the 0·45 μm pore size membrane causes some fractionation of the industrial pectins. However, the fraction removed by the membrane represents only a very low (negligible) mass contribution to the total concentration. For the apple pectin, filtration through a 0·45 μm pore size filter removes the large particles which caused the increase in the light scattering intensity with increasing SEC elution volume for the high elution volume fractions. Whereas filtration of the lemon A pectin sample through a 0·45 μm pore size filter gives almost ideal scattering curves for homogeneous systems, scattering curves remain strongly curved for the lemon B and apple samples. Moreover,

unlike previous results obtained on another citrus pectin (Berth *et al.*, 1990), the initial slope of the scattering curves increases with the elution volume, indicating an increased average radius of gyration.

M_w was calculated from light scattering curves obtained after filtration of SEC fractions through 0·45 μm pore size filters. The two component interpretation (Berth *et al.*, 1990) was applied with the computerized algorithm described by Berth & Lexow (1991) taking Fraction 2 as the model curve for the pure particulate component in order to eliminate the particle contribution in favour of the molecularly dispersed fraction. Although in principle the same considerations with respect to the heterogeneity of the fractions may hold, calculations failed without neglecting the angular region below 45°. Plotting the logarithm of molecular weight against the elution volume (Fig. 8) clearly shows that pectin molecules of identical molecular weight but from different sources elute on SEC at different elution volumes. This definitively demonstrates that SEC is not suitable for the direct determination of the molecular weight of pectins from different sources by the use of a single calibration.

At equivalent elution volumes on SEC, the apple pectin always exhibits higher molecular weights than the two lemon pectins. This is due to two factors: (1) the fractions from the apple pectin contain more NS units than those from the lemon pectins; (2) all fractions from the apple pectin contain large amounts of glucose possibly originating from starch. This supports the importance of neutral sugars emphasized by Berth (1988) and Berth *et al.* (1990).

Figures 6 and 7 demonstrate that preparation of the sample solutions and scattering functions must be carefully considered to get reliable information from light scattering measurements. Indeed, as systems containing traces of particulate matter, pectin solutions are extremely sensitive to any manipulation. For this reason it appears advisable to perform molecular weight determinations with other absolute techniques

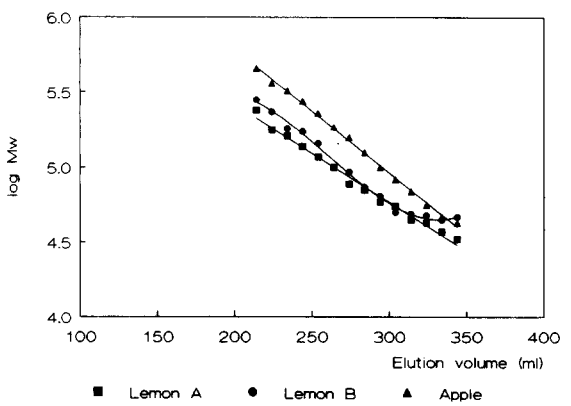

Fig. 8. Change in M_w of the fractions obtained by preparative SEC on Sepharose CL-2B/Sepharose CL-6B as shown in Fig. 2.

such as membrane osmometry and/or sedimentation equilibrium analysis.

CONCLUSION

Chemical analysis of SEC fractions showed that within one pectin preparation, molecules differing in hydrodynamic volume vary in composition. In particular, the largest and the smallest molecules are richer in neutral sugars than those of intermediate size. However, the molecules differ only by the proportion of the same building units. They therefore belong to the same polysaccharide species. Aspinall (1970) described pectins as 'chemically homogeneous polydisperse systems consisting of structurally related molecular species with continuously variable proportions of neutral sugar residues', and Anderson & Stoddart (1966) proposed the term 'heteropolymolecular' to describe such systems. Anyway, industrial pectin preparations are undoubtedly complex mixtures of molecules differing not only in molecular size. They can therefore not be considered as series of homologue polymers.

Light scattering measurements clearly indicate that within one pectin sample, fractions of a given hydrodynamic volume remain highly heterogeneous on the basis of their molecular weight. This clearly indicates the coexistence in industrial pectins of particles of very different shapes. All SEC fractions contain various amounts of a very high molecular weight component. As this high molecular weight fraction has a dominant effect on light scattering measurements, most investigators have tried to remove it by ultracentrifugation (Smith, 1976; Berth *et al.*, 1977; Kawabata & Sawayama, 1977; Jordan & Brant, 1978; Plashchina *et al.*, 1985; Berth, 1988; Sawayama *et al.*, 1988), filtration (Smith, 1976; Jordan & Brant, 1978; Axelos *et al.*, 1987; Hourdet & Muller, 1987) or heating (Smith, 1976; Sawayama *et al.*, 1988), but none of these techniques has been completely successful. However, it is evident that the use of filters or any other 'purification' procedure is quite likely to affect pectin solutions so that they are no longer representative of the material originally dissolved. Rejecting any amount of sample raises the question as to whether the purified solution can still be related to the properties of the original material. It appears that pectins which are polydisperse with respect to molecular weight are also polydisperse with respect to the conformation and/or reactivity of molecules. It is now a question of how these particular features affect the physical behaviour of industrial pectins.

The nature of this high molecular weight component is not yet definitively elucidated. As the amount of particulate component appears to increase with increasing quantity of total neutral sugars in the early SEC fractions, Berth (1988) and Berth *et al.* (1990) suggested that these particulate components might be

molecules rich in neutral sugars (i.e. highly branched). This is in agreement with the view that molecules with very different molecular weight but of similar hydrodynamic volume may coelute on SEC. Brigand *et al.* (1990) further suggested that starch is probably the main reason for the excess of light scattering. However, free neutral polysaccharides including starch have been shown to elute only in the tail of the SEC elution pattern (Kravtchenko *et al.*, 1992), and they therefore may not explain the presence of high molecular weight particles in the high molecular size fractions. The fact that these large particles are at least partly retained by membranes with sizes as large as 0·45 μm strongly suggests that they may be extremely large aggregates instead of molecularly dispersed molecules. It is thus very surprising to find such large particles in almost all SEC fractions. Berth & Lexow (1991) suggested that a mechanism other than simple size exclusion might govern the elution pattern of these high molecular species. In addition, it might also be possible that some of these high molecular weight particles are aggregates which could have been formed after SEC fractionation. Indeed, SEC experiments showed that pectin molecules are prone to aggregation and that aggregates can be disrupted by shear forces. These aggregates may thus have been disrupted during SEC fractionation, their constituent molecules fractionated according to classical SEC theory and reforming on resting before further characterization. This hypothesis is supported by the experiments of Smith (1976) who could still modify the light scattering behaviour of pectin solutions even after 20 filtrations through fresh membranes of the same pore size. Each filtration step might have removed only a part of the aggregates, the other being disrupted by the physical treatment. Indeed, all the purification procedures described above failed probably because they only temporarily disrupt aggregates. Only ultracentrifugation which removes dense particles without shear forces, and ion exchange chromatography, which might remove the molecules responsible for aggregation, may be expected to be efficient. We have obtained strong indications that the tendency for aggregation of certain pectin preparations might be due to the presence of some molecules of low DM (intermolecular distribution). However, other mechanisms of aggregation such as hydrogen bonding or hydrophobic interactions (Davis *et al.*, 1980) may also occur in the absence of calcium ions. The simultaneous occurrence of proteins and phenolic compounds in all SEC fractions might also explain aggregation.

It seems that the application of more and more advanced techniques reveals an increasing complexity in the intermolecular distribution of pectin molecules within industrial preparations. However, the importance of such an heterogeneity in determining the physical behaviour still remains largely unexplained.

Since the intermolecular distribution of methyl esters appears to be of great importance to understand the reactivity and probably the physical behaviour of industrial pectins, the same samples have been fractionated by preparative ion exchange chromatography. The resulting fractions have been extensively analysed in order to establish the distribution of the structural constituents among pectin molecules differing in charge. The results of these investigations are the subject of a following paper.

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