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Studies on the intermolecular distribution of industrial pectins by means of preparative ion-exchange chromatography

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Three industrial high methoxyl pectins have been fractionated by ion-exchange chromatography on a preparative scale. Chemical analysis of the resulting fractions revealed that pectin molecules varying in charge, also differ in composition. Neutral sugars, phenolic and proteinaceous compounds were found to coelute with pectin molecules. The nonuronide material appeared to be associated with all pectin molecules, but mainly attached to those requiring a high ionic strength to be released from the ion exchanger. Moreover, despite large quantitative differences, the distribution of individual neutral sugars among the molecules was found to be very similar for pectins from lemon and apple, indicating a great similarity in structural features. Separation of pectin molecules by ion-exchange chromatography was found to depend on many different chemical parameters such as degree of methoxylation, but, probably also the intramolecular distribution of free carboxyl groups and the presence of phenolics. This renders the interpretation of ion-exchange elution profiles difficult and uncertain.

INTRODUCTION

Fractionation by size exclusion chromatography has repeatedly shown that industrial pectins are not chemically homogeneous (Brigand *et al.*, 1990; Kravtchenko *et al.*, 1992*b*); molecules varying in molecular size also differ in chemical composition. High-performance ion-exchange chromatography (HPIEC) showed that industrial pectins are also heterogeneous with respect to their charge (Kravtchenko *et al.*, 1992*a*). However, HPIEC analysis did not allow a deeper insight into the chemical variations which may accompany differences in molecular charge.

Since the introduction of DEAE-cellulose columns in pectin research by the team of Deuel (Neukom *et al.*, 1960; Heri *et al.*, 1961*a*, 1961*b*), ion-exchange chromatography (IEC) has been used by almost all investigators of pectin structure. The basic principle is that separation is achieved according to the charges of pectin molecules, which depend on the number of dissociated carboxyl groups present on the individual molecules. Basically, pectins can thus be fractionated by IEC according to their degree of methoxylation (Heri *et al.*, 1961*a*; Hatanaka & Ozawa, 1964; van Deventer-Schriemer & Pilnik, 1976; Anger *et al.*, 1977) and their covalently linked neutral sugar content (Heri *et al.*, 1961*a*; Hatanaka & Ozawa, 1966; Anger *et al.*, 1977; de Vries *et al.*, 1981). However, it is likely that the size of the pectin molecules also affects the elution pattern (Heri *et al.*, 1961*a*). Anger *et al.*, (1977) observed that mechanically degraded pectins of similar degrees of methoxylation (DM) elute earlier as the molecular size decreases. This contradicts the assumption of Walker & Saunders (1970), who ascribed the influence of the molecular size on the desorption of pectin to a superimposed size exclusion effect. Interactions other than ionic ones, independent of the degree of methoxylation, but dependent on the size of the molecules, might also be involved (Anger *et al.*, 1977).

IEC has, therefore, been widely used to fractionate pectin molecules in relation to their degree of methoxylation (van Deventer-Schriemer & Pilnik, 1976; Schols *et al.*, 1989). As free neutral polysaccharides are not

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retained by the exchanger at low ionic strength, IEC has also been used to isolate pectic substances from mixtures with other neutral polysaccharides (Aspinall *et al.*, 1968; Ishii, 1981, 1982; Barbier & Thibault, 1982; Thibault, 1983; Rombouts & Thibault, 1986; Berth, 1988). More generally, IEC has been used to characterize the intermolecular distribution of pectin samples, the elution profile being considered as a 'fingerprint' (Smit & Bryant, 1967; Brigand *et al.*, 1990). IEC provided a meaningful indication of changes occurring in stored fruits (Knee, 1970) or of differences between various cell-wall extracts (Rombouts & Thibault, 1986; Thibault, 1988; Renard *et al.*, 1990, 1991). IEC also allows the differentiation of pectins deesterified by different ways; pectins deesterified by acid or alkali appear to be homogeneous on IEC, whereas pectins deesterified by plant pectin-esterase elute in large fractions of various degrees of methoxylation (Heri *et al.*, 1961*b*; Schols *et al.*, 1989).

Diethyl-amino-ethyl (DEAE) linked to various matrices has been the most widely used anion exchanger for the fractionation of pectins. Deuel and coworkers (Neukom *et al.*, 1960; Heri *et al.*, 1961*a*, 1961*b*) as well as many other investigators (e.g. Aspinall & Fanshawe, 1961; Rosik *et al.*, 1962; Hatanaka & Ozawa, 1966; Smit & Bryant, 1967; Knee, 1970; van Deventer-Schriemer & Pilnik, 1976; Anger *et al.*, 1977; Ishii, 1978; de Vries *et al.*, 1981) used DEAE-cellulose columns). Because of the low capacity and the poor flow capacity of cellulose ion exchangers, DEAE linked to other matrices such as microcrystalline cellulose (Anger & Dongowski, 1984; Stevens & Selvendran, 1984*a*; Racape *et al.*, 1987; Saulnier & Thibault, 1987; Thibault, 1988), cross-linked dextrans (Aspinall *et al.*, 1968; de Vries *et al.*, 1981; Ishii, 1981; Stevens & Selvendran, 1984*b*) or cross-linked agarose (Michel *et al.*, 1981; Barbier & Thibault, 1982; Stevens & Selvendran, 1984*b*; Rombouts & Thibault, 1986; Axelos *et al.*, 1989; Renard *et al.*, 1990) have been used with advantage. All these commercially available column materials differ from each other by their flow properties, their ionic capacity and their size exclusion limit, which may be very important for the proper binding of high molecular weight molecules such as pectins. Antal & Toman (1976) introduced diethyl-amino-hydroxypropyl, and Sun *et al.* (1987) used quaternary amino-ethyl as alternative exchangers.

After sample application and adsorption on the ion exchanger, the column is washed with low ionic strength buffer in order to remove the unbound substances. Separation is then obtained by varying the conditions of elution. Some of the earlier investigators (Neukom *et al.*, 1960; Aspinall & Fanshawe, 1961; Hatanaka & Ozawa, 1964, 1966) fractionated pectins by increasing the pH. This procedure can be criticized since, at high pH values, methyl esterified pectins undergo deesterification and depolymerization by β-elimination (Albersheim *et al.*, 1960). Most investigators preferred to elute pectin substances by increasing the concentration of the buffer used as mobile phase at constant pH. Thus, buffers such as phosphate (Heri *et al.*, 1961*b*; Rosik *et al.*, 1962; Smit & Bryant, 1967; Knee, 1973*b*; van Deventer-Schriemer & Pilnik, 1976; Anger *et al.*, 1977; de Vries *et al.*, 1981; Stevens & Selvendran, 1984*a*), acetate (Hatanaka & Ozawa, 1969; Ishii, 1978; Barbier & Thibault, 1982; Stevens & Selvendran 1984*a*; Rombouts & Thibault, 1986), carbonate (Hatanaka & Ozawa, 1964), formate (Aspinall *et al.*, 1969), borate (Neukom *et al.*, 1960; Knee, 1973*a*) and succinate (Renard *et al.*, 1990, 1991) have been used in the pH range 4–6·5. Stevens & Selvendran (1984*b*) increased the ionic strength of the mobile phase with a gradient of NaCl concentration in Na borate buffer, and Racape *et al.* (1987) used a nonbuffered gradient of NaCl. The choice of the buffer is mainly directed by the conditions of stability of pectin molecules. However, some other criteria such as compatibility with further chemical analysis must also be taken into account. Knee (1970) claimed to improve the desorption by adding some EDTA to the mobile phase in order to avoid binding of polyuronides to the matrix by divalent metal ions.

Recently, high-performance chromatographic procedures have been developed, making the IEC analysis much faster. Schols *et al.* (1989) used an MA7P column (Bio-Rad, Richmond, CA) coupled to a UV detector, reading the absorbence at 215 nm. Brigand *et al.* (1990) used a monoQ column (Pharmacia, Uppsala, Sweden) coupled with an on-line analyzer to determine the carbohydrate content of the eluate. Unfortunately, HPIEC does not allow the collection of fractions large enough for further investigation.

This paper deals with the preparative fractionation of industrial pectin samples by IEC and the subsequent chemical analysis of the resulting fractions.

MATERIALS AND METHODS

Pectin samples

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

For the calibration of the IEC column, highly methoxylated pectins were prepared by transesterification (van Deventer-Schriemer & Pilnik, 1976) of lemon B pectin, pectic acid was obtained by alkaline saponification (van Deventer-Schriemer & Pilnik, 1976) of the apple pectin sample, and other pectin standards were commercial apple pectins obtained by industrial acid deesterification (Nelson *et al.*, 1977).

Preparative ion-exchange chromatography

Pectin (500 mg) was applied to a column (23 × 5 cm) of DEAE-sepharose CL6B (Pharmacia, Sweden) equilibrated with 0·005 M Na succinate buffer at pH 4·8. After loading, the column was washed with 0·005 M Na succinate buffer (250 ml) and eluted with a linear gradient from 0·005 to 0·5 M of Na succinate buffer (2000 ml). The gradient was generated by two peristaltic pumps governed by an LCC500 pump controller (Pharmacia, Sweden). Elution was continued with 1000 ml of 0·5 M Na succinate buffer and 250 ml of 0·5 M NaOH. Fractions (20 ml) were collected and assayed for uronic acid and total neutral sugar contents. Fractions were grouped to constitute 12 pools. Each pool was ultrafiltered through PM10 membranes (nominal cut-off of 10 000 daltons; Amicon, Lexington, MA) and freeze-dried prior to further characterization.

High-performance size exclusion chromatography

High performance size exclusion chromatography (HPSEC) was performed as described by Kravtchenko *et al.* (1992*a*).

Chemical analysis

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

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

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). The method was modified as described previously (Kravtchenko *et al.*, 1992*b*).

The protein content was evaluated by the Sedmak & Grossberg assay (1977), using microtiter plates as described by Rylatt & Parish (1982).

Total phenols were estimated with Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) without copper treatment, using ferulic acid as standard (Swain & Hillis, 1959). Folin-Ciocalteu reagent (0·2 ml) was added to the sample solution (*c.* 0·5%), followed, after 5 min, by 0·4 ml of saturated Na_2CO_3 solution. The absorbence at 750 nm was read after 1 h.

RESULTS

Preparative IEC

Large scale IEC was used in order to fractionate industrial pectin samples according to the charge of their constituent molecules. DEAE-Sepharose CL6B (cross-linked agarose) was chosen because of its excellent flow properties, allowing the use of high flow rates, its high exclusion limit (4 × 10^6 daltons) and its high total charge capacity, all resulting in high loading capacity. Sodium succinate buffer was chosen because its pH corresponds to that of maximum stability for pectin molecules. Unlike acetate, succinate buffer does not disturb either the determination of galacturonic acid by the *m*hdp assay or the determination of the degree of acetylation.

Figure 1 shows the elution patterns obtained by IEC of the three industrial pectin samples. For the reasons presented elsewhere (Kravtchenko *et al.*, 1992*b*), the results obtained by colorimetry with orcinol and *m*hdp have not been corrected for mutual interference. The amount of neutral sugars appears thus to be overestimated.

AUA recoveries were measured by determining the

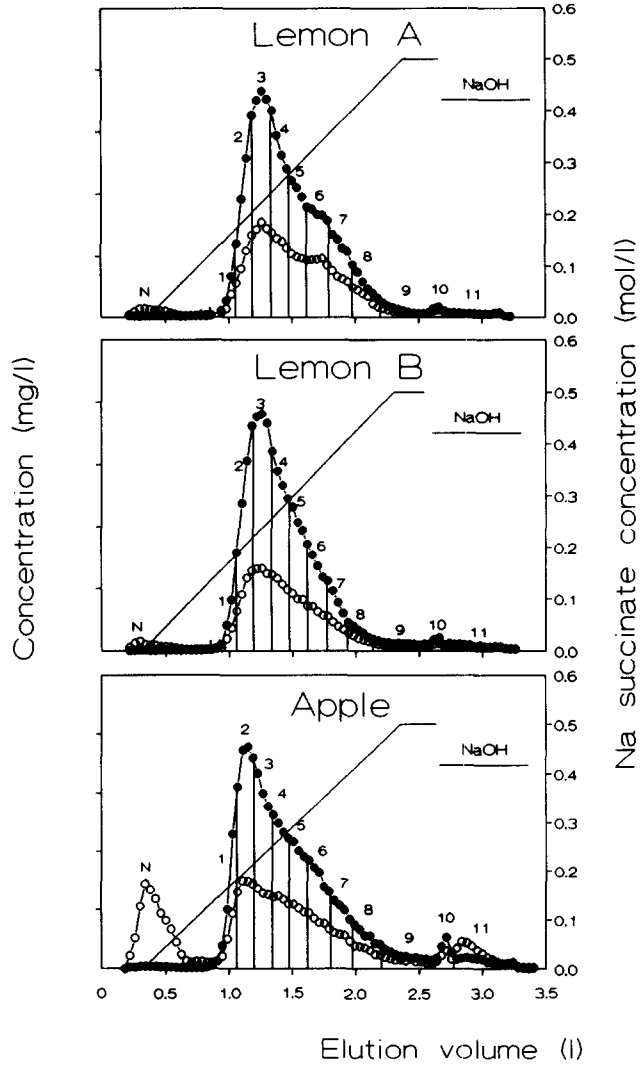

Fig. 1. IEC elution pattern of the three industrial pectin samples on DEAE-Sepharose CL6B. —●—, galacturonic acid; —○—, total neutral sugars.

AUA content in the eluate of 5 ml columns of DEAE-Sepharose CL6B eluted with 0·5 M Na succinate buffer and 0·5 M NaOH after injection of a known amount of pectin. Recoveries were 95, 99 and 89% for the lemon A, lemon B and apple pectins, respectively. Even after washing with NaOH, the top of the column exhibited a brownish coloration, which indicates that some phenolic compounds were bound irreversibly to the column material. This was especially clear for the apple pectin sample, which was found to contain a higher amount of phenolics than the lemon ones (Kravtchenko *et al.*, 1992*a*). Since polyphenols may be attached to some pectin molecules, nonionic binding via phenolic compounds might explain the incomplete recovery of the apple pectin from the IEC column. Indeed, at alkaline pH, the ion exchanger is completely uncharged and ionic interaction cannot be held responsible for the irreversible binding of some uronide material. Polyphenols could only be removed from the IEC column by washing with 0·1 M $NaClO_2$.

A fraction which consisted mainly of neutral sugars (fraction N) is not retained by the column. This fraction represents the 'ballast' (Michel *et al.*, 1981; Brigand *et al.*, 1990) — neutral polysaccharides which are not covalently bound to the pectin molecules. The free neutral polysaccharides represent only a small fraction of the lemon pectin samples, but they are very abundant in the apple pectin sample. This is in agreement with the results derived from copper purification experiments of these same pectin samples (Kravtchenko *et al.*, 1992*a*).

All the galacturonic acid containing material binds to the column at low ionic strength and elutes by raising the buffer concentration. Only a small percentage (fraction 11) requires NaOH to be released. As already observed by HPIEC (Kravtchenko *et al.*, 1992*a*), the broadness of peaks indicates that pectin molecules are distributed over a wide range of DM. Axelos *et al.* (1989) found a similar elution pattern for a commercial high methoxyl pectin sample. The shoulder which occurs on the tail of the main peak (fractions 5–7) can be interpreted as the second peak found with other pectins extracted from apple (Knee, 1970) and sugar beet (Le Quéré *et al.*, 1981).

Coloration with orcinol shows that some neutral sugars coelute with the galacturonide material, confirming that neutral sugars are not only present as a coextract, but are also covalently linked to the galacturonan backbone (Neukom *et al.*, 1960; Ishii, 1978; Dea & Madden, 1986; Kravtchenko *et al.*, 1992*a*).

It is interesting to note that after ultrafiltration and freeze-drying, fractions 6 and 7 from the lemon pectin samples could only be redissolved in water of alkali in the presence of CyDTA. This indicates that pectin molecules of these fractions are associated via polyvalent cations (Grant *et al.*, 1973), which may originate from the buffer solution or the pectin itself.

Molecular size distribution

The fractions obtained by preparative IEC have been rechromatographed using HPSEC. The elution patterns obtained are shown in Fig. 2.

The unbound fractions from the three industrial pectin samples (fraction N) elute relatively late on HPSEC, confirming that free neutral polysaccharides have a much smaller hydrodynamic volume than pectin molecules (Le Quéré *et al.*, 1981; Michel *et al.*, 1981; Brigand *et al.*, 1990; Kravtchenko *et al.*, 1992*a*).

The subsequent acidic fractions elute very early on

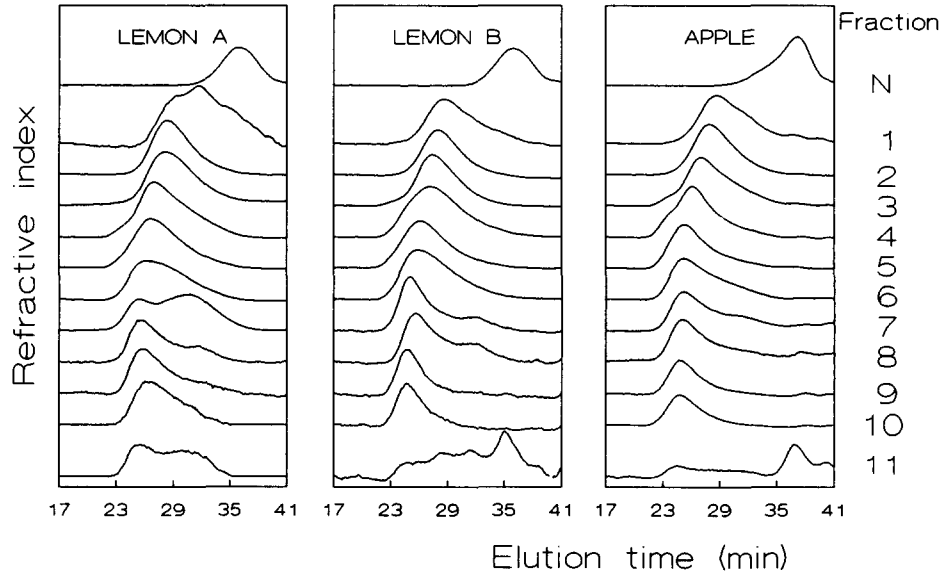

Fig. 2. HPSEC elution pattern of the fractions obtained by preparative IEC of the three industrial pectin samples as indicated in Fig. 1.

HPSEC, indicating that they are constituted of molecules of large hydrodynamic volume. However, fractions 1, 7 and 11 appear to be not homogeneous on SEC. Fraction 1, especially that from the lemon A pectin, appears to be constituted of three partly resolved peaks. Fraction 7 is characterized by the presence of a second peak, which elutes after the main peak. Fraction 11 is mainly constituted of small molecules, which elute at a retention time very similar to that of the neutral fraction (fraction N).

These data do not support those presented by Smit & Bryant (1967). These authors did not find any significant change in the viscosity of IEC fractions from an industrial citrus pectin and thus concluded that the molecular weight is nearly identical for all pectin molecules, irrespective of their charge. From rough IEC fractionation, Sun *et al.* (1987) also could not detect any change in the SEC elution pattern of the IEC fractions.

Neutral sugar distribution

Table 1 shows the sugar composition of the fractions from the three industrial pectin samples which do not bind to the IEC column at low ionic strength (fraction N). All three neutral fractions contain arabinose, galactose, glucose, xylose, fucose and mannose. These neutral polysaccharides may come from the partial solubilization of the other carbohydrates constituting the cell walls from which pectin was extracted, or may have been separated from the pectin molecules by the 'trimming' reaction which occurs during the industrial hot acid treatment (Kravtchenko *et al.*, 1992*a*). The neutral polysaccharides isolated from the two lemon pectin samples are remarkably close to each other, both in quantity and composition. The free neutral polysaccharides from lemon pectins are mainly composed of galactose and arabinose in the ratio 4·8:1. Glucose represents only about 10% of the neutral sugar units, confirming the low starch content of lemon pectins (Kravtchenko *et al.*, 1992*a*). In contrast, the neutral fraction from the apple pectin contains a higher amount of neutral polysaccharides of different composition. The proportion of arabinose is higher whereas that of galactose is much lower (ratio gal/ara 1:2·5). This difference may be due to the presence of arabinogalactans of type I (1,4-linked galactose units) and type II (1,3/1,6-linked galactose units) (Clarke *et al.*, 1979) in different proportions in the free neutral polysaccharides from apple and lemon. In addition, the neutral fraction from the apple sample contains a larger proportion of glucose, but relatively less fucose and mannose than those of the lemon samples. The three pectin samples also contain some rhamnose in their free neutral polysaccharide fraction although only traces of galacturonic acid could be detected. The rhamnose to galacturonic acid ratio is very high (i.e. ratio lies between 0·1 and 0·5). These rhamnose-rich pectin molecules of small size (see above) may be free 'hairy regions', which do not bind to the ion exchanger because of their high neutral sugar content and/or their high DM (de Vries *et al.*, 1982; Saulnier & Thibault, 1987). Indeed, de Vries *et al.* (1982) mentioned that about 10% of the pectin obtained by mild extraction from apple only binds to the IEC column after partial saponification. Molecules with a very high rhamnose/galacturonic acid ratio have also been found in the fraction from the same pectin samples which do not precipitate with copper ions (Kravtchenko *et al.*, 1992*a*).

Table 1. Glycoside composition of the unbound fractions from the three industrial pectin samples, (expressed as wt % of total sugars)

Glycoside	Lemon A	Lemon B	Apple
Galacturonic acid	1·3	0·8	1·0
Rhamnose	0·5	0·5	0·1
Fucose	1·2	1·2	0·3
Arabinose	14·3	13·9	40·2
Xylose	0·6	0·4	0·4
Mannose	5·5	5·5	0·8
Galactose	69·0	66·4	16·1
Glucose	7·5	11·3	41·1

The following fractions (1 to 11), which elute by increasing the ionic strength, contain both galacturonic acid and neutral sugar units, but in varying proportions. According to their total neutral sugar content, as shown in Table 1, pectin molecules can be divided into five distinct groups. Fractions 1, especially that of the lemon A sample, are characterized by a very high neutral sugar content. The total neutral sugar content then reaches a minimum in the fractions 2 to 4, which represent the main bulk of the galacturonic acid of the whole samples. The shoulder which occurs on the tail of the main peak (fractions 5–7) is characterized by a relatively high total neutral sugar content. The total neutral sugar content decreases again in fractions 8 to 10 before increasing dramatically in the fraction eluted by NaOH (fraction 11). This neutral sugar distribution, particularly clear for the lemon pectins, is masked in the apple sample by a constant rapid increase of the glucose content with increasing ionic strength. Such a neutral sugar distribution in the IEC chromatograms is very surprising since the charge of the molecules could be expected to decrease with increasing neutral sugar content. The high amount of glucose in fraction 11 may be due to the presence of retrograded starch molecules which remained on the top of the column until the pH was high enough to allow their solubilization and their subsequent elution. The high neutral sugar content of the pectin fractions eluting at low ionic strength (fractions 1) has already been observed for pectins from tobacco (Sun *et al.*,

1987) and cherry fruits (Barbier & Thibault, 1982). It is possible that these fractions contain some free neutral polysaccharides which can bind to the ion exchanger and require some increase in ionic strength to be released. Indeed, Neukom *et al.* (1960) have been able to fractionate arabans on DEAE-cellulose with phosphate buffer in the range 0·025–0·25 M. This indicates that complete removal of neutral polysaccharides cannot be attained by IEC. Copper precipitation, which is more specific for the presence of uronide, should be preferred. It is interesting to note that despite differences in origin and procedure of extraction, we found that, in the three industrial pectins, the distribution of total neutral sugars was very similar to that reported for other pectin samples (Knee, 1970; Le Quéré *et al.*, 1981; de Vries *et al.*, 1982). Minor discrepancies are probably due to differences in IEC procedures and/or sharpness of the fractions analyzed.

A closer examination of the neutral sugar composition reveals that the relative proportion of the neutral sugar units also varies widely. Figure 3 shows the relative composition of the neutral sugar other than glucose within the IEC fractions from the three industrial pectins. Without taking into account glucose units, all three pectin samples exhibit very similar distribution of the neutral sugar units. The proportion of galactose, which is the major neutral sugar constituent, is at a maximum in fractions 2 and 3, decreases to a minimum in fractions 7 and 8 and then increases again up to fraction 11. The proportion of arabinose is just the opposite to that of galactose and shows maxima in fractions 1 and 5/6. However, it also increases in fraction 11. The proportion of rhamnose units is clearly maximal in the fractions 7 to 9 for the lemon pectins and 6 to 8 for the apple pectin. The proportion of xylose and fucose seems to be almost constant except for a slight minimum in fractions 3 to 6. The proportion of mannose units is clearly higher in the last four fractions with a clear maximum in fraction 10 for the lemon pectins. These data suggest a discontinuous rather than a continuous distribution of neutral sugar units in industrial pectins.

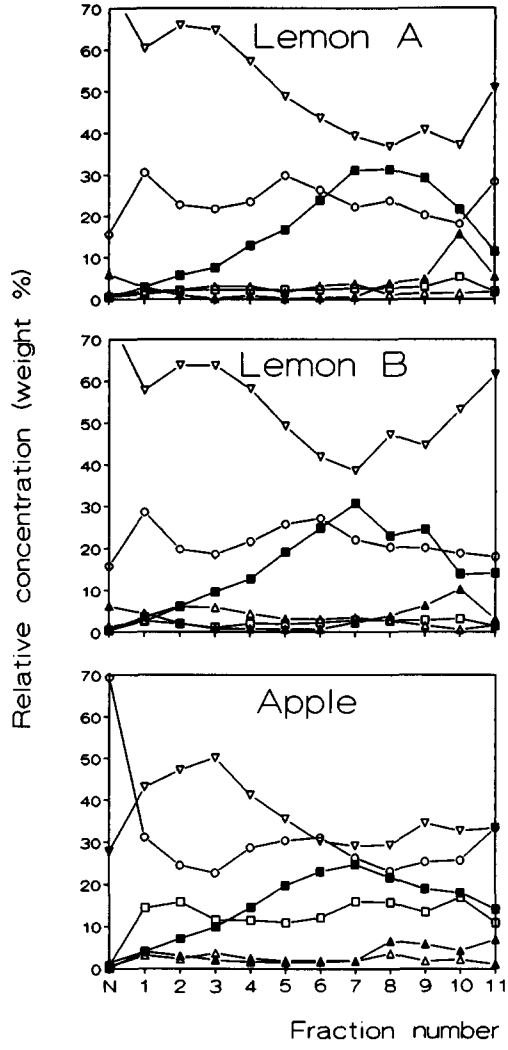

Fig. 3. Relative composition of the neutral sugars other than glucose in the fractions obtained by preparative IEC of the three industrial pectin samples as indicated in Fig. 1. —■—, Rha; —△—, Fuc; —○—, Ara; —□—, Xyl; —▲—, Man; —△—, Gal.

Distribution of proteinaceous and phenolic compounds

Table 2 shows the distribution of phenolic and proteinaceous compounds among the fractions obtained by fractionation of the three industrial pectins by IEC. Phenolics could not be determined in certain lemon pectin fractions because of the presence of CyDTA (see above), which reacts positively with the Folin-Ciocalteu reagent.

Except for lemon B pectin, where a high phenolic content is also found in the first fraction, phenolic compounds appear to coelute mainly with pectin molecules which require a high ionic strength to be released from the IEC column. The apple pectin differs from the lemon pectin samples by a higher phenolic content in fractions 2 to 6. However, in the apple sample, the amount of phenolic compounds present in the IEC fractions is lower than expected from the total phenol content of the unfractionated sample (Kravtchenko *et al.*, 1992*a*). Indeed, phenolic compounds seem to bind very strongly to the column (see above) and some of them may not be recovered.

Proteins exhibit a distribution very similar to that of phenolics. Coelution of proteins and phenolics has already been observed in fractions obtained by SEC of the same industrial samples (Kravtchenko *et al.*, 1992*b*). All the fractions obtained from the apple pectin contain less proteins than the corresponding ones from the lemon pectins. Although the method of estimation is different, these results are in excellent agreement with the total protein contents found previously (Kravtchenko *et al.*, 1992*a*).

Distribution of methyl and acetyl esters

Table 2 shows the DM and the degree of acetylation (DAc) of the fractions obtained by preparative IEC of the three industrial pectins. Since they were eluted by NaOH and therefore at least partly saponified, degrees of esterification of fractions 11 were not determined.

For comparison, the preparative IEC column has been calibrated, using a series of pectins varying in average DM. Since they were produced by acid

Table 2. Chemical composition of the fractions obtained by preparative IEC (expressed as wt % of AUA)

	Lemon A										
	1	2	3	4	5	6	7	8	9	10	11
AUA (%)[a]	2·5	13·8	21·7	17·4	12·9	13·3	9·5	5·3	2·1	0·7	0·8
Rha	0·9	0·2	0·3	1·1	1·6	2·6	3·0	4·7	3·6	3·4	5·0
Fuc	0·6	0·1	0·1	0·2	0·2	0·3	0·4	0·2	0·2	0·3	0·7
Ara	9·0	0·8	0·8	1·8	2·5	2·6	1·9	3·3	2·3	2·6	11·2
Xyl	0·4	0·1	0·1	0·2	0·2	0·2	0·2	0·4	0·3	0·8	0·8
Man	1·0	t	t	t	t	t	0·1	0·7	0·7	2·8	2·7
Gal	21·8	2·7	3·0	5·3	5·0	5·3	4·2	6·2	5·7	6·6	24·6
Glc	1·6	0·1	0·1	0·2	0·2	0·2	0·2	0·8	0·8	3·1	4·9
Total NS	34·7	3·9	4·3	8·5	9·5	11·0	9·6	15·9	13·5	19·2	49·0
DM (%)[b]	83	95	78	79	70	66	52	37	60	59	n.d.
DAc (%)[b]	1·0	0·9	0·9	1·6	1·9	1·9	1·7	1·7	2·1	3·0	n.d.
Phenolics	0·1	0·1	0·1	0·1	0·1	n.d.	n.d.	1·4	1·3	7·0	13·3
Proteins	0·6	—	—	—	0·1	0·2	0·3	1·8	1·2	1·7	13·5
	Lemon B										
	1	2	3	4	5	6	7	8	9	10	11
AUA (%)[a]	3·3	17·4	24·2	18·2	13·7	10·4	6·0	2·8	1·9	1·1	1·0
Rha	0·4	0·3	0·7	1·2	2·3	2·8	4·4	3·1	3·3	1·6	11·1
Fuc	0·3	0·3	0·4	0·5	0·4	0·4	0·5	0·4	0·2	0·1	1·2
Ara	2·7	0·8	1·2	1·9	2·9	2·8	2·9	2·5	2·5	2·0	12·8
Xyl	0·3	0·1	0·1	0·2	0·2	0·2	0·4	0·4	0·4	0·3	0·0
Man	0·5	0·1	0·1	0·1	0·1	0·1	0·4	0·6	1·0	1·3	2·7
Gal	6·7	3·2	4·7	6·2	6·8	5·3	6·3	7·1	6·8	6·8	53·7
Glc	1·3	0·2	0·2	0·2	0·3	0·3	0·8	0·8	1·5	4·2	9·3
Total NS	14·0	4·7	6·9	9·8	12·7	11·5	15·1	14·5	15·5	16·2	90·6
DM (%)[b]	77	95	75	76	64	66	54	35	63	66	n.d.
DAc (%)[b]	1·3	1·0	1·0	2·1	2·3	2·1	1·6	1·4	3·7	3·7	n.d.
Phenolics	1·0	0·2	0·1	0·7	0·1	0·1	n.d.	n.d.	1·6	1·9	10·1
Proteins	0·7	—	—	0·4	0·1	0·2	0·7	1·7	0·6	3·8	9·5
	Apple										
	1	2	3	4	5	6	7	8	9	10	11
AUA (%)[a]	7·3	20·2	17·1	13·5	11·4	11·3	7·2	4·7	3·2	1·9	2·1
Rha	0·4	0·4	0·7	2·0	3·7	4·0	3·9	4·1	3·1	2·2	5·4
Fuc	0·3	0·1	0·3	0·3	0·3	0·3	0·3	0·7	0·3	0·3	0·4
Ara	2·6	1·1	1·6	3·6	5·2	5·0	3·8	4·0	3·8	2·9	11·7
Xyl	1·2	0·7	0·8	1·5	1·9	1·9	2·3	2·7	2·0	1·9	3·8
Man	0·4	0·2	0·2	0·3	0·3	0·3	0·3	1·4	1·1	0·6	3·0
Gal	4·4	2·6	4·1	6·4	7·4	6·0	5·2	6·3	6·3	4·6	14·2
Glc	7·6	2·0	3·9	4·0	5·1	4·7	4·5	7·9	9·6	5·6	112·6
Total NS	16·5	7·0	11·3	17·8	23·5	21·9	20·0	26·5	25·8	17·8	150·6
DM (%)[b]	76	94	72	69	60	58	50	41	58	55	n.d.
DAc (%)[b]	2·0	2·4	3·1	5·0	5·6	5·4	4·2	3·7	6·1	4·3	n.d.
Phenolics	0·4	0·2	0·2	0·3	0·4	0·6	2·3	5·2	1·6	1·2	9·1
Proteins	—	—	—	0·1	—	0·1	0·3	1·6	1·6	1·2	4·8

[a]Fraction % of the whole sample.
[b]Expressed as mol % of AUA.
n.d. Not determined.

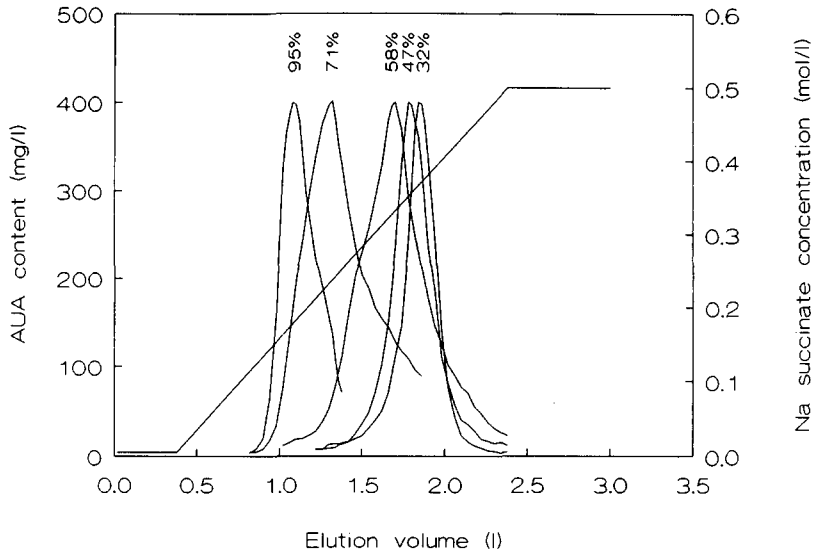

Fig. 4. Calibration of the preparative IEC column with pectin samples of different DM as indicated in percentages.

deesterification in aqueous solution, the distribution of their free carboxylic groups may be considered to be random (Speiser *et al.*, 1947). Figure 4 clearly shows that standard pectins elute in a rather narrow peak, their elution time increasing with decreasing DM.

As expected, the DM of the fractions obtained by IEC of the three industrial samples decreases regularly from fractions 2 to 8. However, except for fractions 2 to 4, the DM appears to be lower than expected from the elution volume of the fraction. This indicates that structural features other than the average DM govern the strength of binding to the ion exchanger. Since all these fractions have similar hydrodynamic volumes, some molecular size effect cannot be assumed responsible for their elution delay. Anger & Dongowski (1984) suggested that this is due to differences in the distribution of the free carboxyl groups along the pectin backbone. A blockwise distribution will result in zones of higher charge density, which bind strongly to the ion exchanger. Moreover, fractions with a DM very different from that expected were found to have a higher content of phenolics. This is especially true for the two last fractions from the three pectin samples. Binding by a nonionic mechanism via phenolics may thus explain that some pectin molecules elute later than expected from their average DM. This hypothesis is reinforced by the fact that the fractions from the apple pectin sample which contain more phenolics than those of the lemon pectin fractions have a lower DM. Another possible cause for the delayed elution of the pectin fractions has been observed during the column calibration. After injection, the pectic acid standard (DM 0%) could not be released from the column even by elution with NaOH. Elution could only be obtained with 0·05 M ammonium oxalate in 0·5 M NaOH, indicating that the pectic acid was insolubilized in the column by reaction with polyvalent cations which occur as impurities in buffer solutions. However, since recoveries are close to 100%, this phenomenon is probably negligible for the IEC fractionation of high methoxyl pectin samples. Average DM has been recalculated for the three pectin samples from the contribution of each of their constituting fractions. For the lemon pectins, recalculated DM was found to be in good agreement with average DM measured on the whole samples (Kravtchenko *et al.*, 1992*a*). For the apple pectin, recalculated DM was found to be 68%, although the average DM was 74%. This indicates that the fraction which remained bound to the IEC column was highly esterified.

On the other hand, all the fractions 1 are less methyl esterified than expected from their order of elution. This is probably due to their high neutral sugar content (Table 2) and/or their small molecular size (Fig. 3).

For the three pectin samples, DAc increases with increasing elution volume on IEC. The variation is not continuous, but seems to progress by steps of increasing DAc: fractions 1–3, 4–9 and 10–11. This particular distribution remains completely unexplained. As expected from the average DAc of the whole samples (Kravtchenko *et al.*, 1992*a*), the fractions from the apple pectin samples are always more acetylated than those of the lemon pectin samples.

CONCLUSION

Large scale preparative IEC revealed that pectic substances from industrial samples varying in charge are heterogeneous with respect to their content of neutral sugars. Such a heterogeneity has already been observed earlier, for instance in pectins from apple (Neukom *et al.*, 1980; de Vries *et al.*, 1981), sugar beet (Le

Quéré *et al.*, 1981), cherry fruit (Barbier & Thibault, 1982) and tobacco (Sun *et al.*, 1987). Both the total content and the relative composition of the neutral sugars vary discontinuously among pectin fractions varying in charge. It is very interesting to note, however, that, despite large differences in total neutral sugar content, the relative proportion of the different neutral sugar units other than glucose exhibits very similar distributions for the three industrial pectins, irrespective of their botanical origin. Moreover, pectin samples are also heterogeneous with respect to their content of phenolic and proteinaceous compounds. Proteinaceous and phenolic compounds coelute mainly with pectin fractions requiring a high ionic strength to be released from the IEC column.

Surprisingly, the last eluting fractions exhibit a high DM as well as a high neutral sugar content and are, therefore, only weakly charged. In fact, the mechanism of IEC of pectic substances appears to be much more complex than generally thought and is still not completely understood. The strength of binding to the column depends on many different parameters, such as average DM and distribution of free carboxyl groups, neutral sugar content, molecular size or phenolic content. Interpretation of the IEC elution patterns is rendered extremely difficult by the complexity of pectin composition and structure. The problem is also accentuated by the fact that some low methoxylated pectin molecules can precipitate inside the column if calcium ions are present. Nonionic adsorption via phenolic compounds and/or precipitation with multivalent cations may be responsible for the incomplete recovery of galacturonic acid observed by many investigators (Thibault, 1983; Saulnier & Thibault, 1987; Renard *et al.*, 1991). Release of calcium pectinate can be achieved by adding a calcium-complexing agent such as oxalic acid or EDTA. Phenolic rich pectin molecules can be released from the ion exchanger by washing the column with $NaClO_2$.

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