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Insight into the Structure of Pectin by High Performance Chromatographic Methods

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ABSTRACT

Various high performance chromatographic methods (analytical and preparative size exclusion chomatography, as well as ion exchange chromatography) have been developed in order to provide a deeper insight into the structure of pectin. The study mainly concentrated on commercial high-methoxy samples and products from the MRS research centre.

By coupling size exclusion chromatography (SEC) and low angle laser light scattering (LALLS), realistic values of the molecular weight and molecular weight distribution can be obtained, as the contribution from aggregates may be easily determined. Differences between apple and lime pectin are shown.

Preparative size exclusion chromatography allowed us to obtain information about the dependence of pectin composition on molecular weight by analysing the neutral sugars, the degree of esterification and the acetyl content in each individual molecular weight fraction. It was found that the high content of abrabinose and galactose in the low molecular weight fractions was associated with the ballast of free polysaccharides that accompany the actual pectic chain in all natural pectins.

High resolution ion exchange chromatography was used to compare the intermolecular distribution of degrees of esterification (DE). Distributions of DE were found to be very narrow, with very few exceptions and by analysing SEC fractions, they were found to be constant with the molecular weight in all cases.

INTRODUCTION

The chemical structure of pectin and its molecular weight distribution (MWD) has been the subject of many scientific studies. Recently, an explosion in pectin research has occurred (Fishman & Jen, 1986) with more than 200 references cited in 1983 and 1984. Although pectin polysaccharides are matrix components in the cell wall of all higher plants, industrial sources are restricted to apple pomace and citrus fruit peels. Pectins are mainly composed of 1, 4 linked α-D-galacturonic acid (GA), either free (FGA) or in the esterified form. Neutral monosaccharides are found in almost all pectin preparations. These include rhamnose which is believed to be in the polysaccharide backbone, and galactose, arabinose, glucose and xylose, which are present either as side chains or as part of a few % of extraneous neutral polysaccharides.

Of those laboratories working on pectin, some are mainly concerned with the elucidation of the fine structure of the molecule, by using enzymic or chemical degradation of the chain and subsequent analysis of the residual fragments (de Vries *et al.*, 1982; Thibault, 1983; de Vries *et al.*, 1983*a*, 1984; Kohn *et al.*, 1985; Rombouts & Thibault, 1986). Conductimetry (Thibault & Rinaudo, 1985) and other techniques are sometimes used for the same purpose. As a result of these studies it has been known for some years that the neutral sugar side chains exist in blocks giving rise to 'smooth' and 'hairy' regions along the pectic chain.

Similar studies have been developed to establish the intra- and intermolecular distribution of the methoxy group (de Vries *et al.*, 1983*b*).

Ion exchange chromatography on Deae gels (sepharose or cellulose) using either sodium acetate (Rouau & Thibault, 1984) or phosphate (de Vries *et al.*, 1981; Anger & Dongowski, 1984) buffers is the method used to routinely separate pectin from its neutral ballast as well as to compare the distribution of methoxyl groups.

If gel filtration on classical packings has been used successfully for many years as a purification and/or analytical method, the precise measurement of the molecular weight has always been a challenge. Light scattering experiments on dilute solutions (Jordan & Brant, 1978; Berth *et al.*, 1982; Plashchina *et al.*, 1985; Chapman *et al.*, 1987; Hourdet & Muller, 1987) as well as measurements of colligative properties show non-ideal behaviour with the occurrence of aggregates, microgel structures and multichain associations. These phenomena add to the well-established difficulties which arise in the chromatography of such polyelectrolytes (Masuda *et al.*, 1979). For some years, attention has been focused on the use of high performance size exclusion chromatography (SEC) to obtain information on pectin molecular

weights (Barth, 1980; Fishman *et al.*, 1984; Deckers *et al.*, 1986; Fishman *et al.*, 1987). Various calibration procedures have been described, related to the universal concept proposed by Benoît (Grubisic *et al.*, 1967). Size exclusion chromatography coupled to continuous viscosity measurement has even been described recently (Fishman, 1987).

High performance SEC of polysaccharides has been in the process of development for some years in our laboratory. Analytical SEC results on carrageenans (Lecacheux *et al.*, 1985) and various microbial polysaccharides (Lecacheux *et al.*, 1986) were obtained by coupling SEC and a low angle laser light scattering (LALLS) detector.

A large scale preparative SEC system has been built recently (Lecacheux & Brigand, 1988) to provide relatively narrow molecular weight fractions of various polysaccharides in gram quantities. This can be used to obtain a better knowledge of polysaccharide fine structure, and determine the relationship between various functional properties and molecular weight.

These two systems, along with a brand new chromatographic system for the ion exchange chromatography of pectin (IEC), have been used to study the structure and composition of commercial, high-methoxyl pectins. This report is an overview of results that have been progressively obtained over the past 3–4 years. Part of it was presented on a poster at the recent meeting in Grenoble on plant polysaccharides (Lecacheux *et al.*, 1987).

EXPERIMENTAL

Samples

The samples used were commercial, high-methoxy pectins from apple or lime. They were obtained either from Mero-Rousselot-Satia (MRS) or from other major producers.

The material was usually analysed as received, with the exception of formulated products for which sucrose included for standardisation was removed by ultrafiltration.

Analytical size exclusion chromatography

As previously described (Lecacheux *et al.*, 1985; Lecacheux *et al.*, 1986), the SEC chromatograph consists of the following components: a

Waters 6000 A solvent delivery system, a Waters U6K injector and a Jobin Yvon Iota differential refractometer (RI). A set of two Toyosoda columns — PW 6000 + PW 4000 — was used (each 60 cm in length). The whole system was thermostated to 30°C with a Lauda electronically controlled water bath. A Chromatix CMX 100 LALLS detector was inserted on line between the columns and the refractometer. It was equipped with its own temperature control device. Water and pectin solutions were carefully filtered through Millipore membranes. The eluent was 0·1 M $NaNO_3$ (+0·2 g/l NaN_3 as a bactericide) at 1 ml/min. Columns were calibrated using a set of polyethylene oxide standards from Toyosoda. A Chromatix KMX 16 differential refractometer was used to determine dn/dc values under the conditions selected for SEC analysis, i.e. 0·1 M $NaNO_3$. The value obtained (0·150) was the average of 3–4 measurements.

Preparative size exclusion chromatography

The system which was designed in house has already been described (Lecacheux & Brigand, 1988).

An old Waters GPC 200 apparatus, which had been out of use for many years, was totally renewed for this purpose. A Waters 590 EEF solvent delivery system (0–80 ml/min) was used in connection with a home-made pulse dampener. A home-designed injector was operated by three electrovalves. The sample loop (200 ml) could be fed with any volume by means of a 50 ml syringe. Column effluent was monitored by a Waters R 403 refractometer and fractions were collected by using a LKB 2211 system equipped with the Superrac kit.

A set of two stainless steel columns (each 100 mm i.d. × 600 mm) were installed in the oven compartment. They were packed by the classic slurry technique, one with Toyosoda HW 55S gel, the other with HW 75S.

The eluent was 0·1 M $NaNO_3$, the flow rate was 500 ml/h and the system was operated at room temperature. One gram (~200 ml of concentration 5 g/litre) was injected and 200–500 ml fractions were collected. Several injections were often combined to obtain the required quantity. After ultrafiltration (Amicon SIY spiral cartridge), the solute was recovered by isopropanol precipitation, washing and constant weight drying in an oven at 60°C. The absolute molecular weight distribution of the fractions was established by analytical SEC, then they were chemically analysed. Figure 1 gives an example of an RI preparative trace.

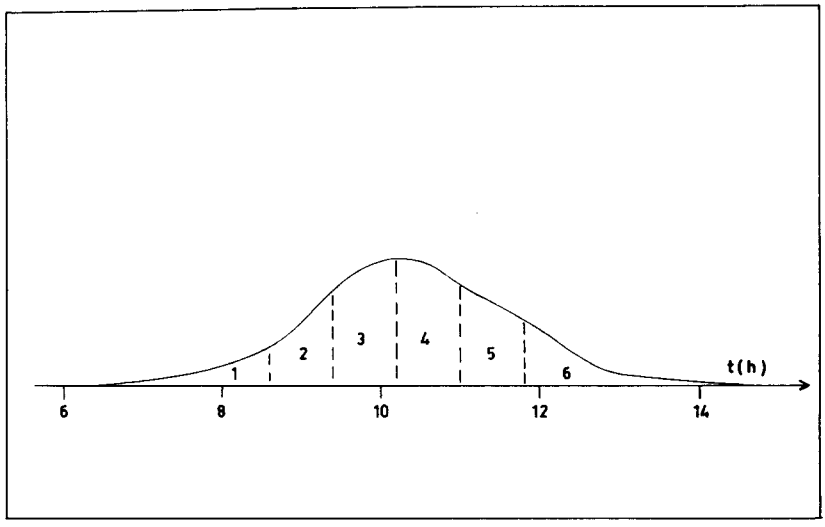

Fig. 1. Preparative RI trace of a lime pectin and slicing of collected fractions (injected quantity 0·72 g).

Ion exchange chromatography

The IEC chromatograph consisted of the following components: a Waters 600 E multisolvent delivery system equipped with a Waters U6K injector, and parts of a Biotronik LC 6000 E chromatograph (post column derivatisation and photometer) as a detector. The column was a Mono Q HR 5/5 anion exchanger from Pharmacia (5 mm i.d. × 50 mm). It was equilibrated with 0·05 M sodium acetate buffer (pH 4·8) at a flow rate of 1 ml/min. Five minutes after the pectin sample (0·1 ml at 2 g/litre) was injected, a linear sodium acetate gradient (from 0·05 to 1·2 M in 35 min at pH 4·8) was applied to progressively elute the bound material.

At the outlet of the column, the effluent was mixed with a 1 g/litre solution of orcinol in pure sulphuric acid at the same flow rate, by using a tee union. The mixture was allowed to react in a teflon loop (0·5 mm i.d. × 20 m) thermostated at 100° in boiling water. The pectin profile was established by monitoring the absorbance at 440 nm on a Shimatzu CR 3A integrator.

Chemical analysis

Neutral sugars were individually determined by ion exchange chromatography in borate buffers using the Biotronik LC 5000 instrument. Of the sample 50 mg were hydrolysed for 8 h at 100°C in 1 M H_2SO_4.

The degree of esterification was determined by potentiometric titration of a free carboxylic group (FGA) (50 mg of the sample was purified for this purpose). In calculating the degree of esterification the percentage of galacturonic acid was obtained by subtracting the percentage of neutral sugars from 100.

For the acetyl content, 25 mg of the sample were dissolved in 5 ml of 0·1 M NaOH. After hydrolysis at room temperature for 1 h, the mixture was neutralised by adding HCl and diluted with water in a 25 ml volumetric flask. The acetic acid content was determined by ion exclusion chromatography (IEC) using a Dionex 14 chromatograph and a conductimetric detector.

Viscosity measurements

Viscosity average molecular weight of samples was determined using a procedure similar to that described by Christensen (1954). A 0·1 g sample of washed pectin was weighed into a 50 ml beaker. After moistening with a few drops of ethanol, the sample was dissolved in about 30 ml of 1% sodium hexametaphosphate previously adjusted to pH 4·5 by the addition of HCl solution. The pH of the pectin solution was then adjusted to pH 4·5 with either NaOH or HCl. The mixture was transferred quantitatively to a 100 ml volumetric flask and made up to volume with the phosphate solution. After standing overnight, the solution was filtered through Whatman No. 41 ashless filter paper. Twenty millilitres of the filtrate were then diluted with 20 ml of hexametaphosphate solution, and the flow time of 5 ml of this solution was measured at 20°C using a No. 150 Ostwald–Cannon–Fenske viscosimeter. The viscosimeter was kept in a water bath at 20°C for 15 min before readings were taken. In the calculations, an average of five readings was used. The viscosity of a 1% hexametaphosphate solution was measured under the same conditions. The viscosity average molecular weight was calculated using the following formula:

$$\text{relative viscosity: } \eta_r = \frac{ts}{to}$$

$$\text{molecular weight: } M_\eta = \frac{(\eta_r^{1/6} - 1)^6}{C \times K}$$

where $K = 4{\cdot}7 \times 10^{-5}$; ts = the flow time of pectin solution; to = the flow time of 1% sodium hexametaphosphate solution; C = concentration in terms of g galacturonic acid/100 ml.

RESULTS

Molecular weight distribution by analytical SEC

Pectin chains are known to be at their most stable in solution near pH 5. Before choosing 0·1 M $NaNO_3$ as the SEC eluent a comparison of results was made with those obtained using a 0·1 M acetate buffer, pH 4·8, as the mobile phase. As the chromatograms (RI and LALLS) were identical, sodium nitrate was chosen for SEC of nearly all the polyelectrolyte studies. A typical chromatogram is given in Fig. 2.

Some specific features were found after analysing a large number of samples. For the sake of clarity, only a selection of them is given in Table 1. A comparison has been made between three values of the molecular weight: M_η the value deduced from the viscosity of the sample as previously described, M_{POE} the weight-average molecular weight obtained from the RI trace using a calibration with polyethylene oxide and M_w the weight-average molecular weight given by the LALLS detector (it is supposed to be an absolute value). As the ratio of M_η to M_{POE} is nearly constant ($M_\eta/M_{POE} = 0{\cdot}31$) with a standard deviation less than 10%), it is easy to conclude that these two methods of measuring the molecular weights of pectins will rank the samples in the same order. Despite the fact that they are not absolute methods, it seems that there are no major drawbacks in using either viscosity or the RT trace from SEC to compare products of various origins. In contrast, M_w from light-scattering seems to be inconsistent.

Weight-average molecular weight values range from 0·4 to 2·6 and seem to be higher for apple (M_{POE}) pectin than for lime pectin. A mean value of 0·5 with a reasonable standard deviation still emerges for samples from citrus fruit peels. This gives the relationship $M_w = 1/2\ M_{POE}$, a rule that will be suggested henceforth. Higher values are supposed to be due to an 'excess' of light scattered by aggregates which tend to be found more in pectins from apple pomace.

Coupling of SEC and LALLS is a well-known method of detecting aggregates in a polymer solution. Most of the time, a peak on the LALLS output comes before the main peak, although there is no material visible on the RI trace. Its contribution to the M_w summation may be easily removed. An example of this is given for hydroxy ethyl cellulose in Fig. 3. Pectins have never exhibited a peak such as this during the present authors' study. It must therefore be concluded that an excess of light is present all along the main chromatogram. To support this hypothesis, a comparison was made of the calibration curves in pectin units that can be established for each injection using the concurrent signals, as we

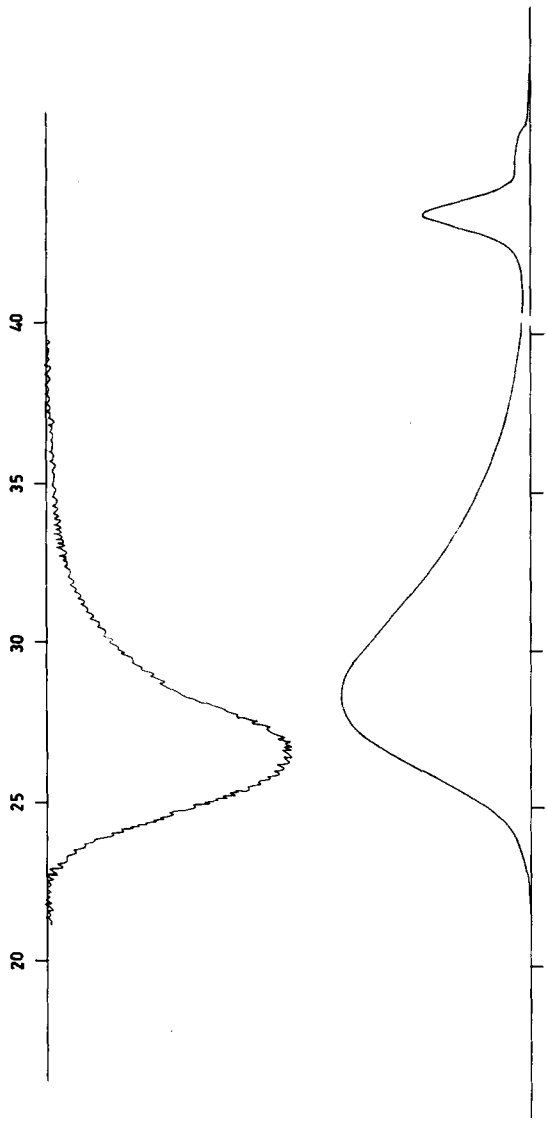

Fig. 2. Analytical SEC of a pectin sample: RI (lower trace) and LALLS (upper trace). (Eluent 0·1 M $NaNO_3$ at 1 ml/min and 30°C, injected quantity 0·4 mg.)

TABLE 1
Molecular Weight (in thousands) and Polydispersity for a Selection of Pectins

Sample	*Origin*	M_η	M_{POE}	M_w	$\frac{M_\eta}{M_{POE}}$	$\frac{M_w}{M_{POE}}$	I
1	Apple	115	365	350	0·31	0·96	7·4
2	pectin	90	280	200	0·32	0·70	10
3	ex.	55	175	460	0·32	2·64	6·9
4	MRS	155	415	425	0·38	1·02	6·6
5	Lime	115	410	240	0·28	0·58	3·9
6	pectin	110	360	165	0·30	0·45	4·6
7	ex.	150	430	205	0·35	0·47	3·7
8	MRS	125	400	250	0·31	0·62	4·5
9	Lime	140	455	235	0·31	0·51	5·7
10	pectin	170	555	250	0·31	0·46	4·2
11	from other suppliers	85	305	120	0·28	0·39	4

previously described for carrageenans (Lecacheux *et al.*, 1985). For apple pectins, a systematic upwards shift in molecular weight is found, compared to the curve for lime pectins, when only one calibration should exist. A possible explanation is proposed in the next paragraph.

The last column in Table 1 is the polydispersity I

$$I = \frac{M_w}{W_n}$$

deduced from the POE calibration. For this parameter, it is well-known and it has been directly demonstrated (Prochazka & Kratochvil, 1987) that the use of RI and LALLS signals is not the most suitable procedure. The samples selected illustrate the general tendency we have observed for apple pectins to be more polydisperse than lime pectins. This difference between the two families is confirmed by preparative SEC experiments.

Results obtained by preparative SEC

Interesting information about the variation in pectin composition with molecular weight distribution of polymers is given from preparative fractionation by SEC followed by comparative analysis of fractions. The

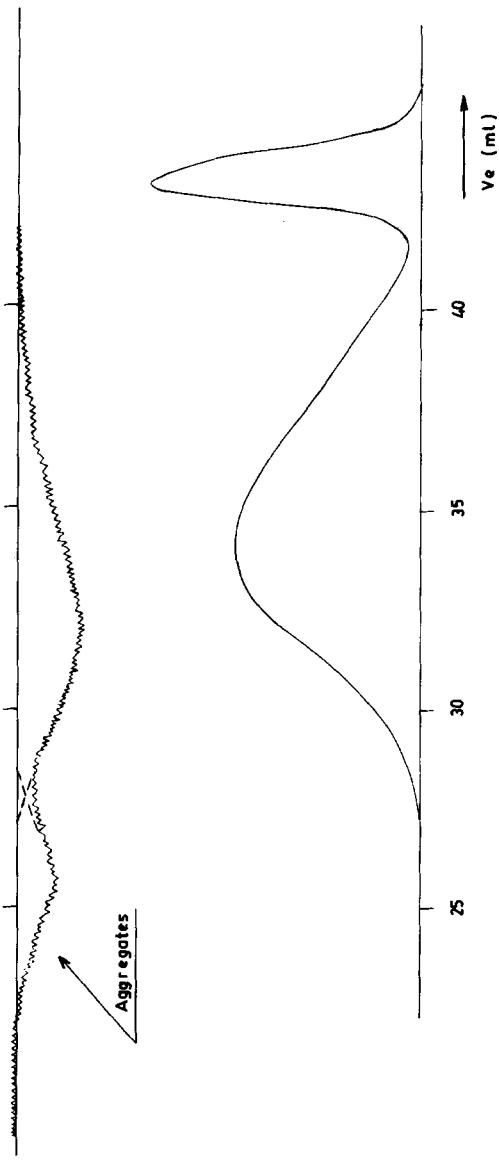

Fig. 3. Evidence of aggregates in hydroxy ethyl cellulose solution (0·1 M LiCl at 1 ml/min, 60°C, injected quantity 0·4 mg). RI (lower trace) and LALLS (upper trace).

information obtained about the neutral sugars in pectins is illustrated in Fig. 4. Note that samples were analysed without prior clean up.

In all cases, the rhamnose and xylose content showed the least variation with molecular weight. The rhamnose content slightly increased with M_w in the range of 1–2%. The xylose content of apple pectins showed a similar molecular weight dependence but the overall content was lower.

Only traces of xylose ($\simeq 0{\cdot}1\%$) are found in samples from citrus origin. The main feature of the galactose and arabinose contents is their

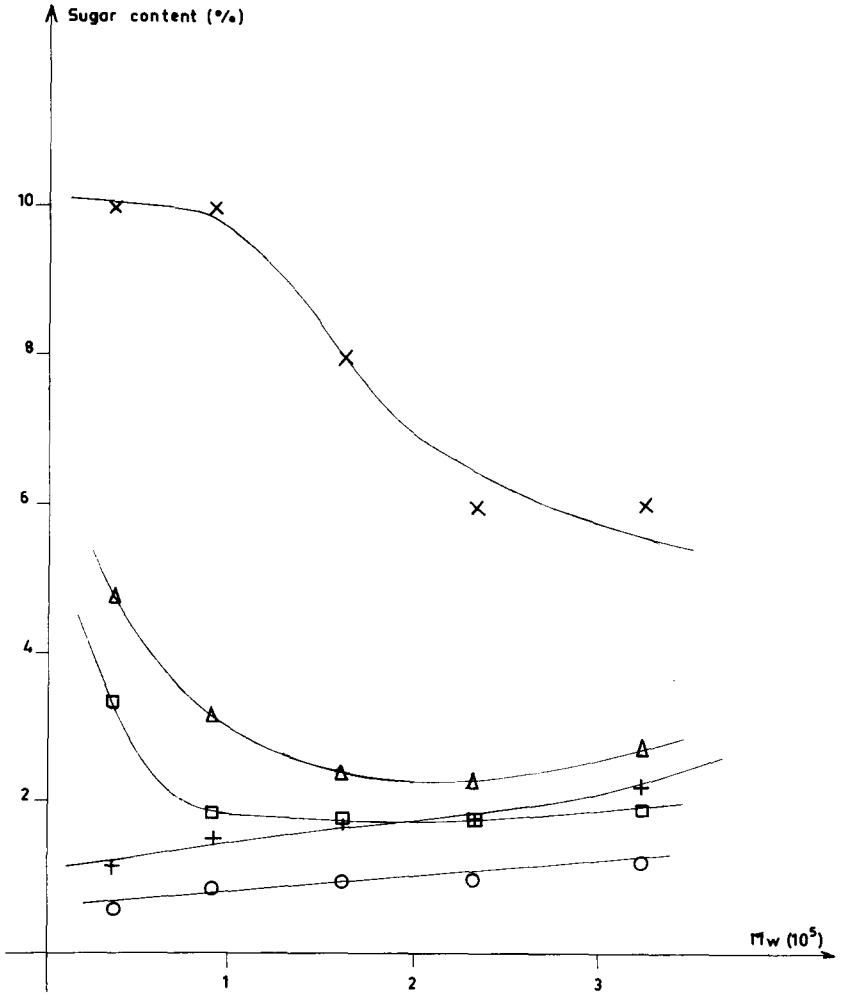

Fig. 4. Neutral sugars against molecular weight for an apple pectin (—×— glucose, —△— galactose, —+— rhamnose, —○— xylose, —□— arabinose).

unexpected increase in the low molecular weight region, sometimes with values as high as 20% while their molar ratio to rhamnose is near unity at higher molecular weight. The glucose content, from starch, may reveal in pectins' origin. Significant values of a few up to 10% were only found in apple pomace pectins. It is present all along the MWD with some decrease with molecular weight. The differences that were shown by analytical SEC between apple and lime pectin can now be traced to a possible origin. Starch is probably the main reason for the excess of light all along the MWD as it is well-known for its ability to form aggregates. Its high content in the low molecular weight tail could account for a broadening of the MWD, resulting in higher I values. When purified from neutral ballast on Deae gels, apple pectins lose almost all their glucose and the increase in galactose and arabinose content with decreasing molecular weight is not observed.

The ballast can thus be precisely defined: arabans and galactans of low molecular weight and specifically for apple pectins starch of wide MWD.

The variation of the acetyl content with molecular weight was only established on a few samples. As can be seen from Fig. 5, a certain

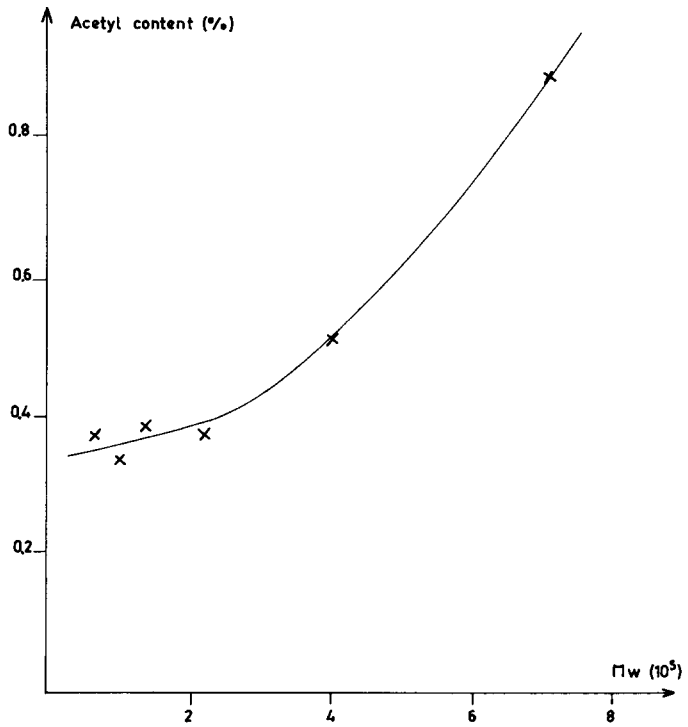

Fig. 5. Acetyl content in a lime pectin as a function of molecular weight.

increase with molecular weight is found. The curve seems to resemble that of galactose. As far as DE is concerned, only slight changes were observed with molecular weight. Figure 6 gives a typical result obtained for lime pectin.

DE increases slightly with molecular weight from 72% to 77%. Decrease with molecular weight has been found in apple pectins but the accuracy is not high enough to come to any definite conclusions, except that changes, if any, are of limited magnitude. All the results for one sample have been put together in Table 2. Figures 1, 5 and 6 relate to this sample.

Distribution of DE by IEC

Figure 7 provides a qualitative calibration of our system with three samples of different DE. If all neutral species are eluted at the void volume of the system (5 ml), retention times of pectins are clearly related to their DE. Our series of commercial, high-methoxy pectins was analysed using this system. With one exception (see Fig. 8), they all exhibited a narrow DE distribution, with, in some cases, a more or less pronounced tail on the low DE side.

By analysing SEC fractions, it was found that the DE distribution was the same whatever the molecular weight. This was equally the case for the atypical sample with a broad distribution of DE. These results, which agree with results from the previous paragraph, lead to the conclusion

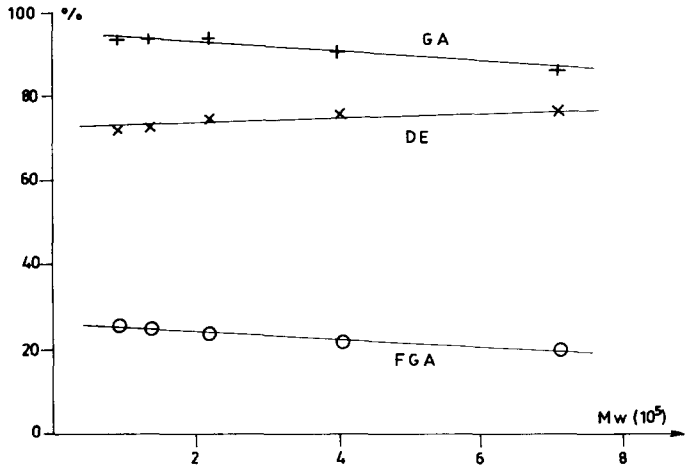

Fig. 6. Changes in the galacturonic acid content (GA), free galacturonic acid (FGA) and the degree of esterification (DE) with molecular weight for a lime pectin.

TABLE 2
Chemical Analysis of Pectin (Sample 9) and its 6 SEC Fractions

Fraction	*Weight fraction (%)*	$M_w \times 10^{-3}$	*I*	*Rhamnose (%)*	*Arabinose (%)*	*Galactose (%)*	*Glucose (%)*	*Total sugars (%)*	*Ga (%)*	*Fga (%)*	*De (%)*	*Acetyl (%)*
(Whole sample)	—	235	6	1·7	2	3·4	0·3	7·4	92·6	21·8	76·5	0·41
1	6	700	3·5	3	3·2	7·2	0·2	13·6	86·4	19·7	77·2	0·87
2	20	395	3	2·1	2·25	4·75	0·05	9·2	90·8	21·6	76·2	0·50
3	28	215	3	1·5	1·5	3	0	6	94	23·4	75·1	0·37
4	26	130	3	1·35	1·5	2·9	0	5·8	94·2	25·3	73·1	0·39
5	16	85	3	1·45	1·7	3·1	0	6·2	93·8	25·7	72·3	0·34
6	4	65	3·5	1·55	2·1	3·7	0·05	7·4	92·6	—	—	0·37

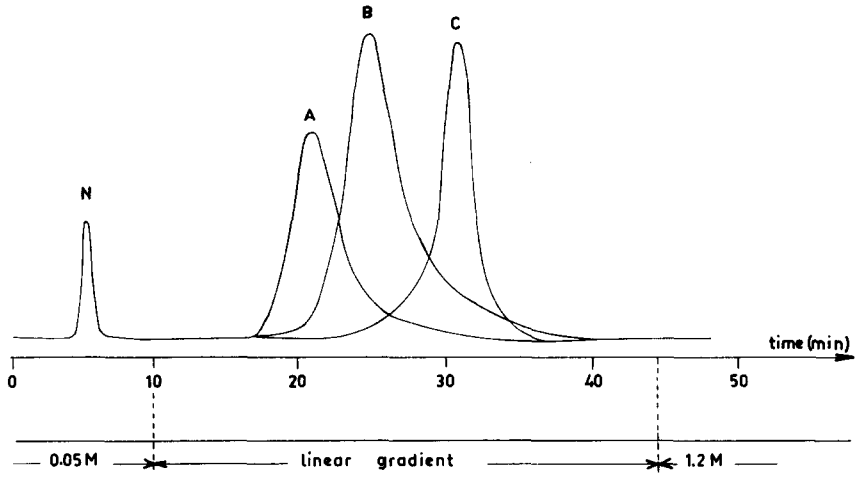

Fig. 7. Calibration of the IEC system using 3 pectin samples with different degrees of esterification. A (DE ≃ 72), B (DE ≃ 60), C (DE ≃ 35) (N = neutral species).

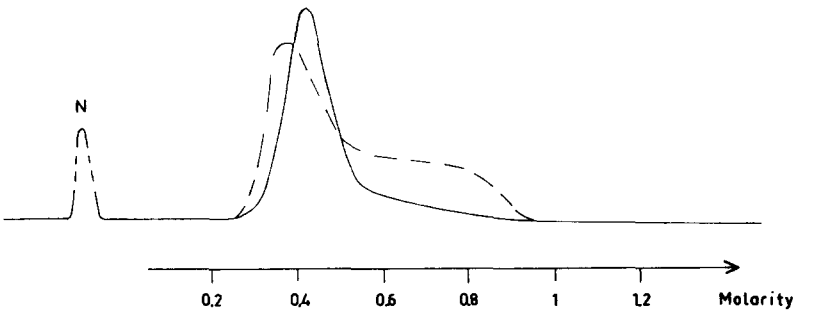

Fig. 8. Comparison of DE distributions for high methoxyl pectins obtained by IEC: —— usual case and - - - the specific sample of broader distribution.

that fractionation of pectin, by SEC then IEC, achieves little, unless the intramolecular distribution of DE masks changes in molecular weight which is unlikely.

CONCLUSION

This study of some commercial, high-methoxy pectins, has led to various results. Size exclusion chromatography/low angle laser light scattering is a valuable means of analysing the molecular weight distribution of any pectin. A direct measurement of absolute molecular weights M_w by

LALLS is not possible, but an empirical rule is proposed to get realistic values:

$$M_w = 0{\cdot}5\ M_{POE}$$

Starch is probably the substance most responsible for an extraneous scattering of light in apple pectin solutions, and could also account for the broadening of the MWD. Commercial pectins have a molecular weight M_w in the range 150·000–280·000. Although 35 years old, the Christensen relationship provides results of the same order of magnitude. A M_η value of 0·62 M_w was obtained in this narrow range of molecular weight. There are araban and galactan chains as free polysaccharides, but they are in the low molecular weight region. In contrast to the neutral sugars and acetyl content, the DE is nearly constant all along the MWD. Intermolecular distributions of DE appear to be relatively narrow (with one exception at the present time) which consequently makes a cross-fractionation of pectin (M, then DE) inappropriate.

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