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Studies on the Heterogeneity of Citrus Pectin by Gel Permeation Chromatography on Sepharose 2 B/Sepharose 4 B

Gisela Berth

Central Institute of Nutrition, GDR Academy of Sciences, Potsdam-Rehbrücke, GDR

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SUMMARY

Commercial high methoxyl citrus pectin was purified by ultracentrifugation or ion exchange chromatography prior to GPC on Sepharose 2 B/Sepharose 4 B. The molecular weights by light scattering and the intrinsic viscosities of the fractions were determined. The effects of the purification procedures on the heterogeneity in shape are discussed by combining the Mark–Houwink plots with the universal calibration plot and the elution profile, respectively. Pectin fractions prepared by GPC are investigated in the same manner. GPC cannot differentiate between lower molecular weight extended coils and more compact high molecular weight molecules of increased polymer density. They are eluted together within a broad elution volume range.

INTRODUCTION

Gel permeation chromatography (GPC) on Sepharose 2 B/Sepharose 4 B is a suitable technique for fractionating different pectins according to their hydrodynamic volumes (Anger & Berth, 1985, 1986). It was shown from measurements of the intrinsic viscosities $[\eta]$ and the molecular weights $\bar{M}_w$ from light scattering of the fractions obtained from GPC that the shape of the elution curve does not relate simply to the molecular weight distribution of the pectin. It was found that the majority of the pectin that occurs is molecularly dispersed in solution but there is a very high molecular weight component with an intrinsic viscosity which is independent of molecular weight. The combination of the $[\eta]$–M relation with the universal calibration line and the elution curve, respectively, allow the amount of the latter to be estimated. It was found to be 14% of

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the total in the case of sunflower pectin (Anger & Berth, 1985) and 18% in the case of purified citrus pectin (Anger & Berth, 1986).

It is known from light scattering measurements that pectin solutions contain a microgel component (Smith, 1976; Smith & Stainsby, 1977; Kawabata & Sawayama, 1977; Berth *et al.*, 1977; Jordan & Brant, 1978; Berth *et al.*, 1982; Plashchina *et al.*, 1985). Because of its disturbing influence Berth *et al.* (1977) tried to separate the microgel component from high-methoxyl apple pectin by ion exchange chromatography on DEAE-cellulose and ultracentrifugation. According to Plashchina *et al.* (1985) the microgel component is removed quantitatively by preparative ultracentrifugation of pectin solutions of concentrations below 0.3–0.5%.

During his ultracentrifuge studies on pectins Devine (1974) observed the presence of a rapidly sedimenting pectinacious material of high molecular weight (several millions) which could not be removed by ultracentrifugation and/or filtration through membrane filters of pore size 0.45 μm. The reported molecular weights for Sunkist citrus pectin were dependent on the centrifugation conditions: at lower speeds they were higher (171 000) than at higher speeds (70 000). The quantity of the high molecular weight tail was found to be very small and in agreement with Plashchina *et al.* (1985), while according to Anger & Berth (1985, 1986) only about 80–85% of the whole pectin shows the typical behaviour expected for a linear homogeneous polymer.

Jordan & Brant (1978) used GPC on Sephadex G-200 to prepare purified pectin fractions. Their experimental results did not allow an unequivocal interpretation in terms of inhomogeneities in molecular weight, size, and shape.

In this paper, firstly, the ultracentrifugation, ion exchange chromatography, and gel permeation chromatography are revised to improve the efficiency with which molecularly dissolved pectin can be separated from the high molecular weight pectin contributing to the 'anomalous' $[\eta]$–M relation. If the separation is complete a linear log $[\eta]$–log M relation over the whole range should be observed in contrast to the original sample where the $[\eta]$–M relation is clearly divided into two sections. To prove this, the elution curves on Sepharose 2 B/Sepharose 4 B, the universal calibration plots (this means log $\{[\eta]M\}$ versus elution volume V_e) and the Mark–Houwink relations are established for fractions which are prepared from a high-methoxyl commercial citrus pectin by ultracentrifugation, ion exchange chromatography, and gel permeation chromatography on Sepharose 2 B/Sepharose 4 B. The effect of these procedures is discussed by comparing the properties of the fractions with the original material.

Secondly, rechromatography under the conditions used for fractionation allows conclusions to be drawn about the heterogeneity of a pectin fraction obtained from a given elution volume from the original elution curve. This is especially important because of the total lack of such information at present.

EXPERIMENTAL

Citrus pectin (Koch-Light, UK) with a degree of esterification of about 60% and a galacturonic acid content of about 70% was used as the source material in these studies.

Gel permeation chromatography: For GPC on Sepharose 2 B/Sepharose 4 B (together about 380 ml) 15 ml of a pectin solution concentration ~2 mg ml^{-1} in 0·037 M pH 6·5 phosphate buffer was used. Fractions of 10 ml each were collected to determine the intrinsic viscosities $[\eta]$ (Viscomatic, FICA, France) and molecular weight $\bar{M}_w$ by light scattering at 90° using an instrument equipped with a helium–neon laser of wavelength 632 nm (Sofica, FICA, France) after filtration through membrane filters of pore size 0·45 μm (Sartorius GmbH, FRG). The pectin concentration within the eluent was monitored continuously using a differential refractometer (Knauer, FRG). Further details have been given elsewhere (Anger & Berth, 1985).

Rechromatography: To give a sufficient quantity of each pectin fraction two neighbouring volume fractions were combined to give 20 ml of volume and the corresponding elution volumes of six to twelve GPC runs were collected. The very dilute pectin solutions were lyophilized or, in some cases, the pectin was precipitated by the addition of a fourfold volume of iso-propanol. To remove the great excess of buffer salt the dried pectin was redissolved at room temperature in distilled water and adjusted to the right buffer concentration by passing the solution through a column of about 120 ml Epidex B 2 (MLW, GDR) with 0·037 M phosphate buffer, pH 6·5 as eluent. The eluent of this column was used directly for GPC on Sepharose 2 B/Sepharose 4 B.

Ion exchange chromatography: The pectin was fractionated on DEAE-Sephacell (Pharmacia, Sweden) using initially 0·005 M phosphate buffer, pH 6·5, followed by a gradient of 0·005 M/0·5 M phosphate buffer, pH 6·5 as eluent (Berth *et al.*, 1977). The first fraction in 0·005 M buffer was discarded. The second fraction (buffer gradient) was desalted to the right buffer concentration on Epidex B 2 for subsequent GPC on Sepharose 2 B/Sepharose 4 B.

Ultracentrifugation: A pectin solution with 5 mg ml^{-1} in 0·037 M phosphate buffer, pH 6·5, was prepared and centrifuged at room temperature for 4 h at 50000 rpm using a preparative ultracentrifuge VAC 602 (MLW, GDR). The supernatant was separated carefully using a syringe and used directly for GPC.

Measurements of the UV spectra: 2 mg of the desalted and lyophilized pectin sample were dissolved in 10 ml of distilled water at room temperature. Five ml of concentrated sulphuric acid were added quickly to 500 μl of the pectinic solution above. The UV spectra were registered with a spectrophotometer (Beckman DK-2A, USA) in the range 360–280 nm against a blank (500 μl distilled water per 5 ml concentrated sulphuric acid). Galacturonic acid (Serva, FRG) and glucose (Leidholdt, GDR) were used as a reference.

RESULTS AND DISCUSSION

The elution profiles of the original pectin and the samples purified by ultracentrifugation and ion exchange chromatography, respectively, on Sepharose 2 B/Sepharose 4 B are shown in Fig. 1(A). Comparing them it is obvious that some high molecular weight material disappears as a result of the purification procedures. This effect is more marked following purification by ultracentrifugation. The loss of pectinic material as a result of ultracentrifugation was not measured exactly. The recoveries from the column were in the range of 90–95% and did not differ significantly from those of the original. In contrast the pectinic fraction obtained from DEAE Sephacell represents only 60–70% of the original material.

Following the universal calibration principle developed by Benoit (Grubisic *et al.*, 1967) the logarithmic product of intrinsic viscosity and molecular weight versus the elution volume V_e was plotted (Fig. 1(B)). One plot held for all three samples. Figure 2 displays their Mark–Houwink plots. These plots are typical of high-methoxyl citrus pectins (Anger & Berth, 1986) and are not modified by the purification of pectin in spite of the different elution profiles of the materials. Following our previous interpretation the purified samples remain heterogenous. The proportions of the high molecular weight material responsible for the anomalous $[\eta]$–M relation can be calculated using the Mark–Houwink plot in combination with the universal calibration plot and the elution profiles, respectively (Anger & Berth, 1985). The point of intersection of the two straight sections of the $[\eta]$–M plot in Fig. 2 is given by the coordinates $\log [\eta] = 2{\cdot}91$(ml/g) and $\log \bar{M}_w = 5{\cdot}43$. This

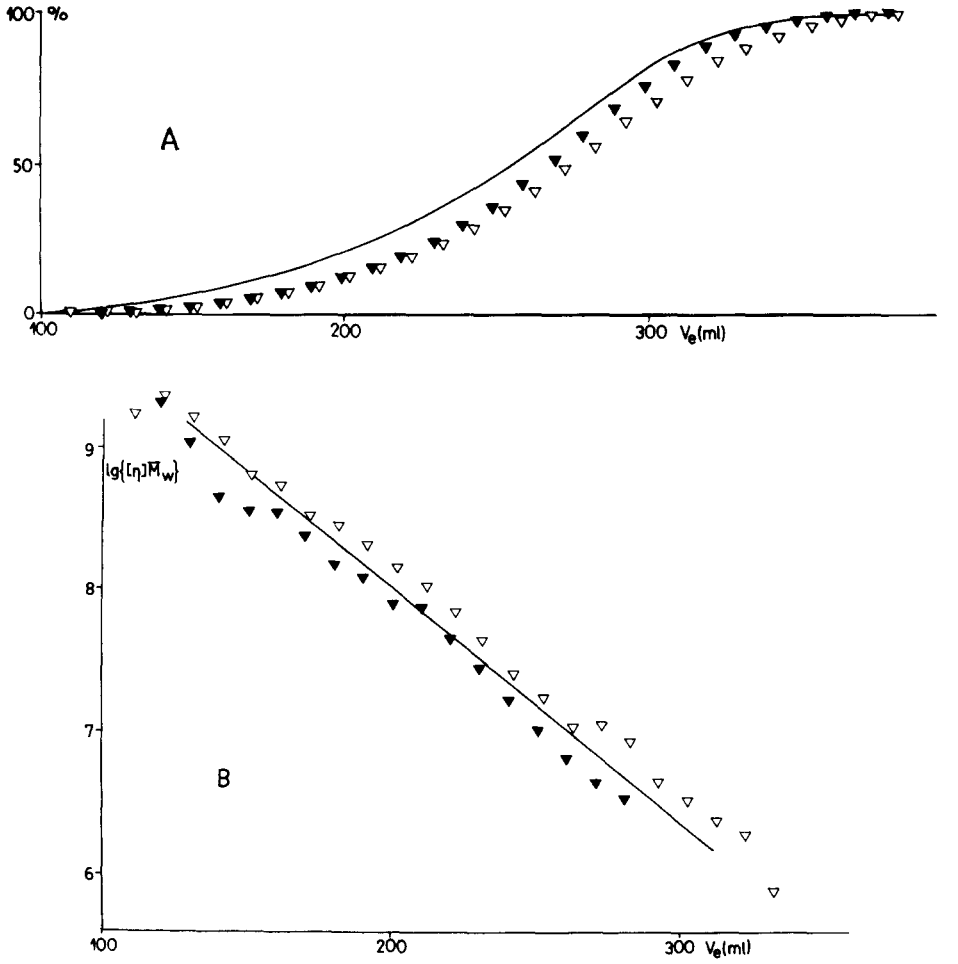

Fig. 1. Cumulative elution profiles (A) and universal calibration plot (B) for commercial citrus pectin (——) purified by ultracentrifugation (▽) or ion exchange chromatography (▼) on Sepharose 2 B/Sepharose 4 B.

means that the proportionality between $\log [\eta]$ and $\log \bar{M}_w$ holds up to $\log \{[\eta]\bar{M}_w\} = 8{\cdot}34$, which corresponds to an elution volume of 180 ml (Fig. 1(B)). The pectin recovered from the column for elution volumes up to 180 ml is high molecular weight material. From Fig. 1 it can be seen that this decreases from about 14% prior to purification to 7% after purification. Obviously purification results in a partial, but not a complete removal of the high molecular weight material. Plashchina *et al.* (1985) reported a loss of pectinic material of about 5% following ultracentrifugation which is in good agreement with our results. As can be

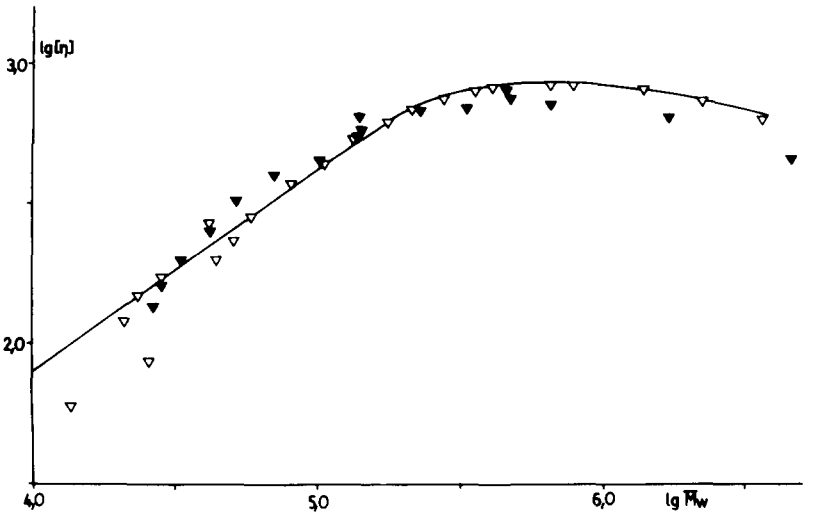

Fig. 2. Mark–Houwink plot for citrus pectin (for symbols see Fig. 1).

TABLE 1
Average Values of Intrinsic Viscosities $[\eta]$ and Molecular Weights, $\bar{M}_w$, of Citrus Pectin Calculated from GPC Fractions

	$[\eta]$(ml/g)	$\bar{M}_w$
Original pectin sample	345	About 3 million
Purified by ultracentrifugation	318	154000
Purified by ion exchange chromatography	326	165000

seen from Table 1 the removal of high molecular weight pectin is evidenced also by the considerable decrease in the average molecular weights while the effect on the intrinsic viscosities is only small.

The average molecular weights in Table 1 confirm very well the results on citrus pectins reported by Devine (1974), and Plashchina *et al.* (1985). Our own studies, however, do not support the conclusions about the polymerhomogeneity of the pectin solutions made by Plashchina *et al.* (1985), but are consistent with the interpretation of Devine (1974). This has an important consequence for the interpretation of light scattering measurements on pectin solutions because of the dominant influence of high molecular weight 'impurities'.

It was now a question of how to prepare pectin fractions free from disturbing high molecular weight material or, in other words, with a

linear $\log[\eta]$–$\log M$ relation without kinking at higher molecular weights. GPC on Sepharose 2 B/Sepharose 4 B was used to produce eight pectin fractions from the commercial citrus pectin. These were rechromatographed on Sepharose 2 B/Sepharose 4 B and measurements of molecular weights by light scattering and intrinsic viscosities were made on fractions within the eluate. In Fig. 3 the integral elution profiles of these fractions are shown. Figures 4 and 5 display the universal calibration plot and Mark–Houwink plot, respectively, for all the samples. To compare these with the original citrus pectin the corresponding data for this material were replotted.

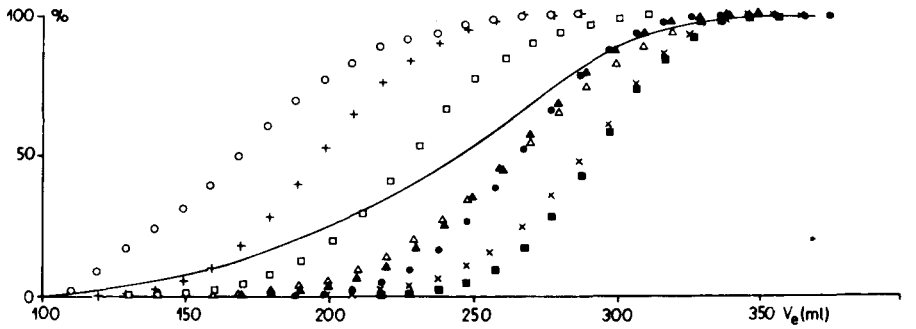

Fig. 3. Cumulative elution profiles of citrus pectin (——) and pectin fractions prepared by chromatography on Sepharose 2 B/Sepharose 4 B. Preparation elution volumes: ○, 150–170 ml; +, 170–190 ml; □, 190–210 ml; △, 210–230 ml; ▲, 230–250 ml; ●, 250–270 ml; ×, 270–290 ml ■, 290–310 ml.

As expected the GPC yields fractions which differ enormously from the original by the enrichment of certain species both in the range of high molecular weights (symbols ○, +, □) and that of smaller molecules (symbols △, ▲, ●, ×, ■). The fractions represented by the latter symbols partly overlap (Fig. 3). The peak maximum for the rechromatography was placed close to the elution volume where the fractions were taken from for only four of the fractions (symbols ○, ●, ×, ■), but was shifted to greater elution volumes for the other ones. This phenomenon depends on the concentration of the solution used for rechromatography and was not observed on more dilute solutions. No irreversible aggregation occurred as a consequence of lyophilization or precipitation.

The universal calibration plot (Fig. 4) shows the groups of values arranged closely to the line of the original. (This 'standard' line differs slightly from Fig. 1(B) because of the interval in time between the

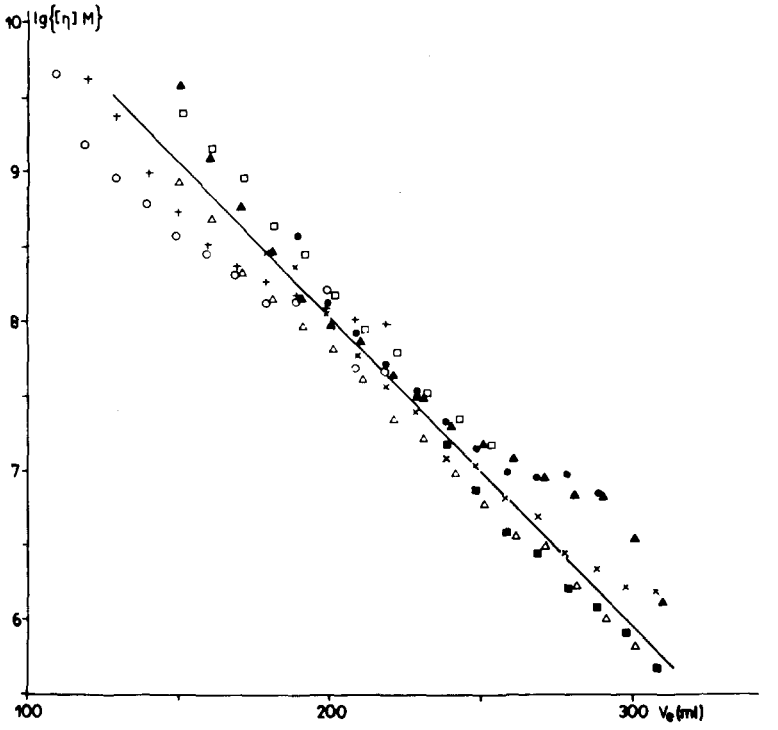

Fig. 4. Universal calibration plot (for symbols see Fig. 3).

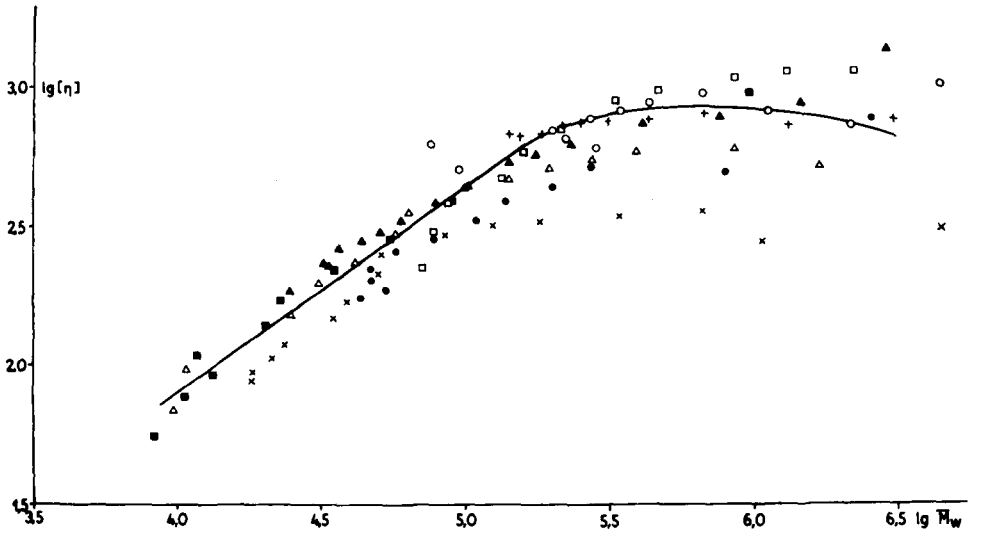

Fig. 5. Mark–Houwink plot (for symbols see Fig. 3).

chromatography runs during which the bed volume will change slightly.) The scatter is higher compared to Fig. 1(B) both at high and low values of elution volume. This is caused mainly by the light scattering data and/or the small polymer concentration. It is difficult to get accurate molecular weights in the case of small molecules, and that is why the deviations here should be considered as being due to experimental error. In contrast high molecular weights can be determined accurately in principle following extrapolation to zero angle. To simplify the subject the angular dependence of light scattering of such pectin fractions (unpublished work, Berth) was neglected in these studies accounting for the deviations at high molecular weights shown in Fig. 4.

The Mark–Houwink plot in Fig. 5 also reflects some scatter in the experimental values, but it follows clearly the form of the original curve which was established from many repeated measurements. If we consider the different fractions (identical symbols) one can recognize the typical shape of the $[\eta]$–M relation in analogy to our previous studies with only one exception (symbol ■). All fractions but this one have a heterogeneous composition. Only this one which represents the lowest molecular weight fraction of the original obeys a linear $\log [\eta]$–$\log M$ relation like a regular homologous polymer series. The intrinsic viscosities at a constant molecular weight in the region of nonproportionality generally decreases with increasing preparative elution volume. This could mean that the molecular density increases with the original elution volume resulting in an underestimate of the molecular weight from GPC at high elution volumes.

The percentages of the high molecular weight component in the fraction were determined as described above using the data in Figs 3–5. The results are shown in Table 2. This was repeated using higher pectin concentrations to improve the accuracy of the light scattering measurements. The elution volumes which were derived from the curvature of the $[\eta]$–M relations differed from the previous run. Only for the first sample (symbol ○) was the former value of 180 ml obtained, but this increased steadily to 215 ml for the last one (symbol ■). Obviously the increased pectin concentrations delayed the carbohydrate elution in agreement with general experience.

If the percentages shown in Table 2 reflect the proportions of high molecular weight material for a normal GPC run (ignoring any deviations on rechromatography caused by concentration effects) we can relate them to the elution volume region where the pectin fractions were taken from for their preparation. This is shown in Fig. 6(A) (points relate to the middle of the preparation elution volume region) which displays the exponential decrease in the level of high molecular weight pectin with

TABLE 2
Percentages of High Molecular Weight Material in the Pectin Samples According to Fig. 3

	Elution volume section (symbol)							
	150–170 ○	*170–190* +	*190–210* □	*210–230* △	*230–250* ▲	*250–270* ●	*270–290* ×	*290–310* ■
Content of high molecular weight material (%)	62	34·5	13	4	2·5	2·5	~ 1	0

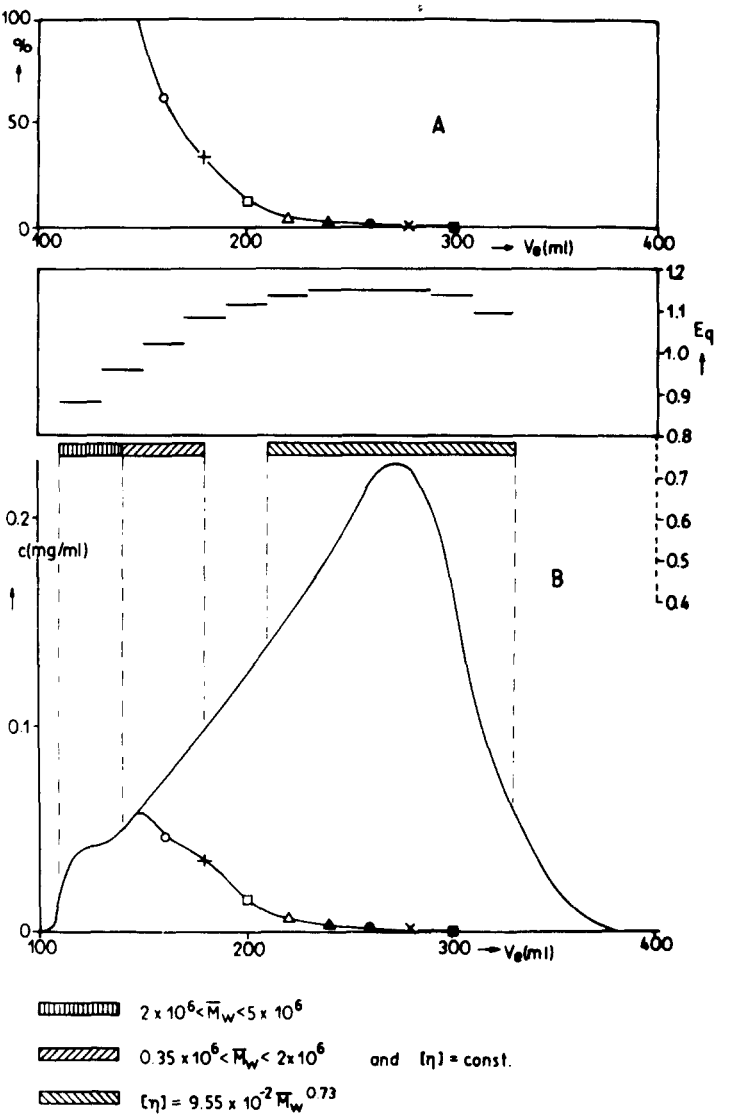

Fig. 6. Percentages of the high molecular weight 'impurity' of samples according to Fig. 3 against the elution volume section used for their preparation (A) and its distribution within the eluate of citrus pectin (B) including the E_q value as a measure for the neutral sugar/galacturonic acid ratio. The shaded regions correspond to the different sections of the $[\eta]$–M plot in Fig. 2.

increasing elution volume. With reference to the original elution profile of the studied citrus pectin these percentages yield the separate elution line for high molecular weight 'impurities' in Fig. 6(B). The area under the latter corresponds to 15% of the total area which is in very good agreement with the value given above.

Up to now differences in the sugar composition of the fractions have not been considered. Previous studies on apple pectins (Bock *et al.*, 1977) showed the enrichment of neutral sugars at the beginning and the end of polysaccharide elution. This was indicated by the UV spectra after adding concentrated sulphuric acid to the carbohydrate solution. Neutral sugars which are present in pectins are characterized by absorption maxima close to 315 nm, but the absorption maximum for galacturonic acid is equal to 298 nm. Introducing the quantity $E_q = E_{298}/E_{315}$, a value of E_q of 1·20 is obtained for pure galacturonic acid and 0·42 for glucose. All values between these limits are obtained for mixtures of galacturonic acid and neutral sugars in different ratios.

These E_q values are shown in Fig. 6(B) together with the elution profile of the citrus pectin including its high molecular weight 'impurities'. They indicate both the overall presence of galacturonic acid units and a steadily rising content of galacturonic acid as the level of high molecular weight 'impurities' decreases. Relatively pure galacturonic acid polymers are found at elution volumes greater than 230 ml. This situation is essentially unaltered after the purification by ultracentrifugation or ion exchange chromatography.

All neutral sugars apart from rhamnose occur as more or less branched side chains along the polygalacturonic acid backbone. Their preferred occurrence within the first 80 ml of the polysaccharide elution could very well explain the curvature of the $[\eta]$–M relationship in Fig. 2. The strongly reduced neutral sugar content in pectin fractions with elution volumes greater than 230 ml seems to justify the assumption of a homologous polymer series and hence the use of the Mark–Houwink equation.

These studies may give an impression of the very complex relations in pectin solutions. The majority of the pectin molecules behaves as extended coils of moderate stiffness and can be described by the equation

$$[\eta] = 9.55 \times 10^{-2} \bar{M}_w^{0.73}$$

up to molecular weights of about 100 000 (Anger & Berth, 1986). With steadily increasing molecular weights the neutral sugar content increases, and the molecules become more and more compact. Only the highest

molecular weight or most compact, the so-called microgel, are probably removed by ultracentrifugation.

For pectin in addition to the usual molecular weight distribution for a polysaccharide, distributions in composition, in shape, and in density have to be considered. This statement is very important if non-colligative methods for polymer characterization are also used. We have never observed processes comparable to those found in amyloses by Husemann *et al.*, 1963 or a time-dependent degradation of the high molecular weight 'impurities'.

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