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The Determination of the Molecular-Weight Distribution of Pectins by Calibrated GPC. Part II: The Universal Calibration

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ABSTRACT

Commercial high-methoxyl citrus and apple pectins were fractionated on Sepharose Cl-2B/Sepharose Cl-4B. To establish the universal calibration line, viscosity and light-scattering measurements were performed on the eluent. The angular dependencies of the scattered light were interpreted in terms of a two-component system to eliminate the contribution of particle scattering at low angles. The galacturonic acid–neutral sugar ratio within the fractions was observed by UV spectra. Results allow conclusions to be drawn on the heterogeneity of each pectin. An approach is suggested for the estimation of heterogeneity in terms of three components.

INTRODUCTION

In studying the separation of pectins by GPC and using light-scattering for polymer characterisation, we are faced with the problem of heterogeneity in addition to the usual molecular-weight distribution. Only an interpretation of the light-scattering angular dependence in terms of a two-component system gave molecular weights for the major component which were consistent with those derived from membrane osmometry (Berth *et al.*, 1990) and sedimentation analysis (Harding *et al.*, 1990). This was shown for the special case of a commercial high-methoxyl-citrus pectin from Koch-Light Ltd (UK). A more general procedure is the universal calibration according to Benoit (Grubisic *et al.*, 1967) for which the logarithm of the hydrodynamic volume $\log\{[\eta]M\}$ must be plotted against the elution volume V_e. A unique straight line should

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result for all the samples studied. The universal calibration was already our previous intention (Anger & Berth, 1986). However, a revision seems to be necessary in the context of the improved discussion of the light-scattering behaviour which considers now the angular dependence between 30° and 150° instead of only one point out of the wide angle range.

For this purpose seven commercial high-methoxyl pectins (four citrus pectins, three apple pectins) were fractionated on a Sepharose Cl-2B/Sepharose Cl-4B column combination and the intrinsic viscosities $[\eta]$ of the eluted fractions determined. From the forms of the $\log[\eta]$ against the elution volume plots, three pectins were selected and the molecular weights of the fractions determined from the angular dependence of light-scattering according to the algorithm previously described (Berth *et al.*, 1990) and then used to establish the universal calibration line. Extinction coefficients E_q derived from the UV spectra after addition of sulphuric acid allow conclusions to be drawn about the neutral sugar–galacturonic acid ratio of the pectinacious fractions obtained from the different pectins.

EXPERIMENTAL

Seven commercial high-methoxyl pectins were studied without purification prior to GPC: citrus pectin: Koch-Light Ltd (UK); citrus pectin type A: Genu (Denmark); citrus pectin type 1400: Grinsted; citrus pectin type 140: Bulmer Ltd (UK); apple pectin type NSSU: Herbstreith (FRG); apple pectin type NS-2: Herbstreith (FRG); and apple pectin type NL-G: Herbstreith (FRG).

They were fractionated on two coupled 200-ml columns (Pharmacia, Sweden) packed with Sepharose Cl-2B and Sepharose Cl-4B. 0·037 M phosphate buffer, pH 6·5, with 0·001 M Na_2EDTA and free from azide was used as eluent and solvent.

For each GPC run, 30 mg pectin in 15 ml buffer were dissolved at room temperature. The polysaccharide concentration was monitored continuously with a differential refractometer (Knauer, FRG). Fractions of about 10 ml were taken for subsequent viscosity (Viscomatic, Fica, France) and light-scattering measurements (Sofica, Fica, France). The light-scattering photometer was equipped with a helium–neon laser (Zeiss, Jena, FRG). Details are given elsewhere (Berth *et al.*, 1990).

E_q was the ratio of the absorbances at 297 and 315 nm from UV spectra observed for the pectin fraction following treatment with sulphuric acid. Two adjacent fractions from the column were pooled and 2·5 ml concentrated sulphuric acid were added rapidly to 500 μl eluent. After cooling to room temperature the UV spectra were measured against a blank on a Beckman DK-2A spectrophotometer (Berth, 1988).

To calculate the particle-free scattering curves from the experimental data a computer program (Simon's basic; Commodore C 64, FRG) was developed. The iteration procedure was as follows and is illustrated in the flow diagram below (Fig. 1).

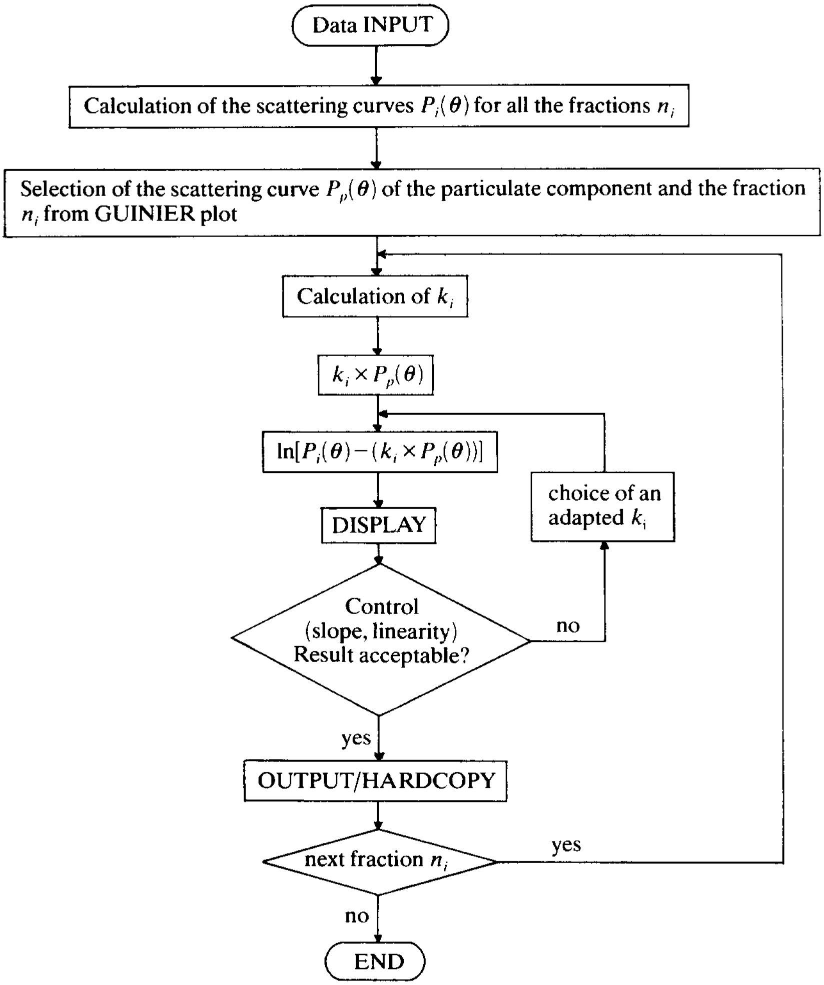

Fig. 1. Flow diagram of the iteration procedure.

First step: calculation of $k'_{\mathrm{i},0}$

$$\frac{\left(\frac{R_{30^\circ}}{K.c}-\frac{R_{120^\circ}}{K.c}\right)'_{\mathrm{i}}}{\left(\frac{R_{30^\circ}}{K.c}\right)'_{2}}=k'_{\mathrm{i},0}$$

where R, K and c have their usual meaning (Huglin, 1972) and an apostrophe is used for the original values. The index 2 is the fraction number which consists entirely of particles. The index i stands for the ith fraction.

Second step: calculation of $\left(\frac{R_\theta}{K.c}\right)_{\mathrm{i}}$

$$\left(\frac{R_\theta}{Kc}\right)'_{\mathrm{i}}-k'_{\mathrm{i},0}\left(\frac{R_\theta}{Kc}\right)'_{2}=\left(\frac{R_\theta}{K.c}\right)_{\mathrm{i}}$$

where the term $\left(\frac{R_\theta}{Kc}\right)_{\mathrm{i}}$ relates to the particle-free component of fraction i.

Third step: control of $\ln\left(\frac{R_\theta}{Kc}\right)_{\mathrm{i}}$ with regard to linearity and slope as required by theory.

Fourth step: variation of k'_{i} until $\ln\left(\frac{R_\theta}{Kc}\right)_{\mathrm{i}}$ fulfils all requirements.

RESULTS AND DISCUSSION

The elution profiles of the seven citrus and apple pectins on Sepharose Cl-2B/Sepharose Cl-4B are shown in Fig. 2. The polysaccharide elution covers the whole range between void and total volume of the GPC column in all cases. Genu citrus pectin and Herbstreith apple pectins type NS-2 and NL-G are diluted with low-molecular sugars. They do not interfere with these studies. The recoveries of macromolecular carbohydrate are given in Table 1 together with the intrinsic viscosities $[\eta]$.

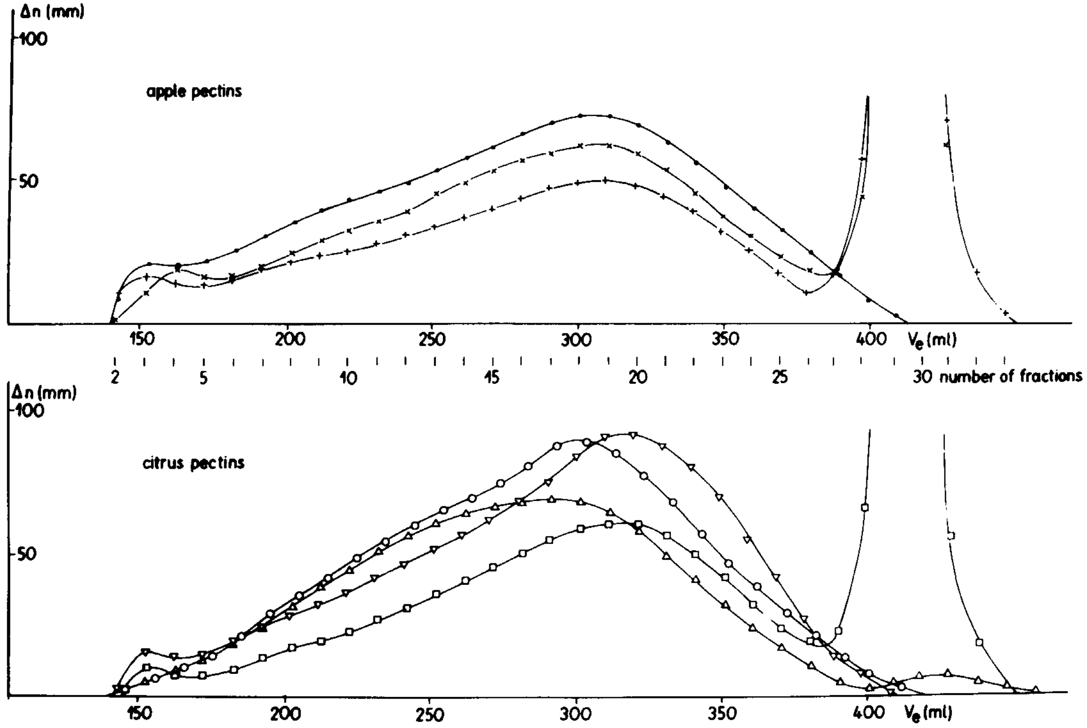

Fig. 2. Elution lines of different high-methoxyl citrus and apple pectins on Sepharose Cl-2B/Sepharose Cl-4B apple pectins: NSSU (●); NL-G (+); NS-2 (×) citrus pectins: Koch-Light (▽); Bulmer (○); Genu (□); Grinsted (△).

TABLE 1
List of Pectin Samples Used, Recoveries of Macromolecular Carbohydrate at GPC and Average Intrinsic Viscosities [η]

Sample	*Producer*	*Recovery (%)*	*[η] (ml g^{-1})*
Citrus pectin	Koch-Light Laboratories Ltd, UK	95	354
Citrus pectin	Bulmer Ltd, UK	88	633
Citrus pectin type A	Genu, Denmark	61	347
Citrus pectin	Grinsted, Denmark	77	648
Apple pectin type NSSU	Herbstreith, FRG	90	440
Apple pectin type NS-2	Herbstreith, FRG	71	397
Apple pectin type NL-G	Herbstreith, FRG	54	403

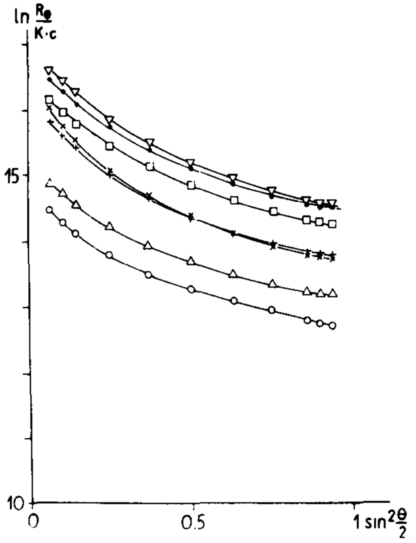

Fig. 3. Guinier plot of solutions with 1 mg ml^{-1} (for symbols see Fig. 2).

The latter were calculated as weight-average values from Fig. 2 and Fig. 4 excluding the sugar additives. They are always in good agreement with the experimental [η] values of the originals if the recoveries from GPC are taken into account.

The angular dependencies of the scattered light at concentrations of 1 mg ml^{-1} are shown as Guinier plots in Fig. 3 (Kerker, 1969). In spite of considerable differences in the level, the shapes of the scattering curves are very similar to each other suggesting all samples are heterogenous like the Koch-Light citrus pectin and contain, to different extents, spherical particles of very high molecular weights which are rich in neutral sugars (Berth *et al.*, 1989). Thus Fig. 3 is indicative of a high percentage of this particle component in Koch-Light citrus pectin and Herbstreith apple pectin type NSSU at the top of the series while Bulmer citrus pectin at the bottom is expected to be reasonably poor in it. This implies that the non particulate macromolecular part of the different pectin samples contributes equally to the light-scattering intensities measured.

Figure 4 displays the intrinsic viscosities of the fractions plotted on a logarithmic scale against V_e. All pectin samples are characterised by the same non-linear trend at significantly different levels. Pectin samples which were estimated as being rich in the particulate component because their high light-scattering intensities in general have a low $[\eta]$ at a given elution volume. The following studies concentrate on three pectins with large differences in the proportion of particulate material. The GPC fractions from Bulmer and Koch-Light citrus pectins and Herbstreith apple pectin type NSSU were studied by light-scattering between 30° and 150°. The results for the two citrus pectins were equivalent to those previously reported (Berth *et al.*, 1990). The scattering curves of apple pectin became more and more flat with increasing V_e (Fig. 5). The two-component interpretation was applied taking fraction 2 as the model curve for the pure particulate component. Comparable GPC fractions derived from Dextran T 2000 (Pharmacia, Sweden) gave strictly linear Guinier plots with slight but steadily increasing negative slopes at molecular weights above $6 \cdot 10^5$ (G. Berth, unpublished). In contrast to our

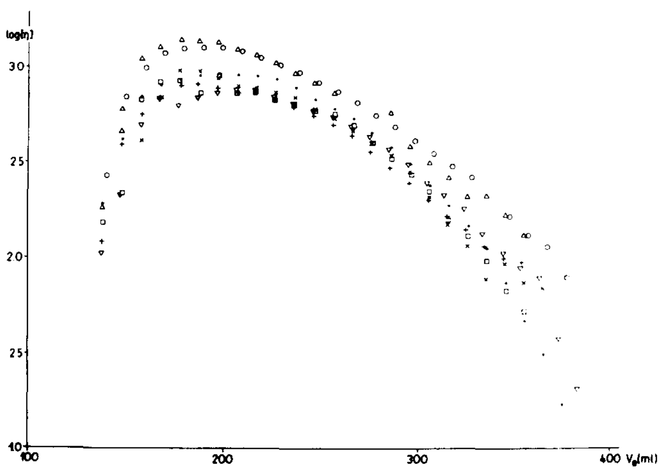

Fig. 4. Log$[\eta]$ against the elution volume (for symbols see Fig. 2).

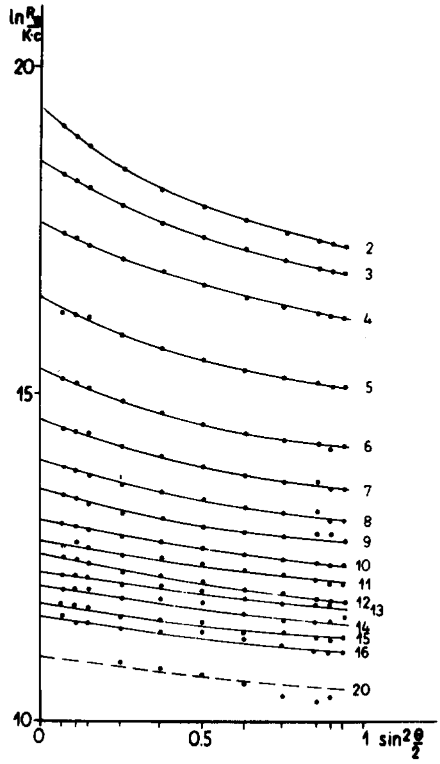

Fig. 5. Guinier plot of the apple pectin type NSSU fractions according to Fig. 2.

previous assertions a slight negative slope of the calculated scattering curves was accepted if the molecular weights of the particle-free component exceeded values of $2 \cdot 10^5$ and the corresponding ZIMM plot gave reasonably linear angular dependencies and radii of gyration which were consistent with random coil polymers (Berth *et al.*, 1990). Table 2 summarises the light-scattering data on the three pectins. $\bar{M}_w$ stands for the molecular weights of the molecularly dispersed major component obtained as a result of our two-component interpretation, k_i' is obtained with respect to the eluent concentrations and the normalized k_i' factors are a measure of the mass contribution of the particulate component. It is seen that the particulate component is concentrated in the void volume. Its contribution reduces rapidly to far less than 1% in fraction 6. The decreasing k_i values coincide with increasing $[\eta]$ values in this region which is consistent with the results from light-scattering with the assumptions made, and the viscosity measurements. Subsequent GPC fractions contain only traces of the particulate component and these do not need to be taken into account in plots of $\log[\eta]$ against V_e.

TABLE 2

Factors k_i' from Two-component Calculations, Mass Contribution k_i of the Particle Component, Molecular Weights $\bar{M}_w$ of the Molecularly Dispersed Pectin Fraction and Intrinsic Viscosities $[\eta]$

Number of fract.	*Apple pectin type NSSU (Herbstreith)*				*Citrus pectin (Koch-Light)*				*Citrus pectin (Bulmer)*			
	k_i'	k_i	$\bar{M}_w\times10^{-3}$	$[\eta]$ $(ml\,g^{-1})$	k_i'	k_i	$\bar{M}_w\times10^{-3}$	$[\eta]$ $(ml\,g^{-1})$	k_i'	k_i	$\bar{M}_w\times10^{-3}$	$[\eta]$ $(ml\,g^{-1})$
2[a]	—	1·00		189·6	—	1·00		104·7	—	1·00		264·7
3	$3·51\times10^{-1}$	$1·46\times10^{-1}$	29 037	414·7	$2·79\times10^{-1}$	$5·21\times10^{-2}$	71 495	211·4	$2·48\times10^{-1}$	$1·08\times10^{-1}$	55 316	683·3
4	$1·34\times10^{-1}$	$5·75\times10^{-2}$	14 395	684·8	$1·76\times10^{-1}$	$3·57\times10^{-2}$	53 063	490·8	$8·00\times10^{-2}$	$2·29\times10^{-2}$	10 147	966·8
5	$3·53\times10^{-2}$	$1·40\times10^{-2}$	5 397	789·2	$6·40\times10^{-2}$	$1·24\times10^{-2}$	5 940	677·0	$5·50\times10^{-2}$	$1·14\times10^{-2}$	1 923	1 157·3
6	$1·32\times10^{-2}$	$4·49\times10^{-3}$	1 761	834·7	$1·70\times10^{-2}$	$2·51\times10^{-3}$	2 295	620·6	$1·75\times10^{-2}$	$2·49\times10^{-3}$	890·3	1 226·5
7	$5·46\times10^{-3}$	$1·52\times10^{-3}$	1 013	876·3	$6·20\times10^{-3}$	$7·23\times10^{-4}$	975·5	676·0	$8·60\times10^{-3}$	$8·90\times10^{-4}$	439·6	1 231·5
8	$2·66\times10^{-3}$	$6·46\times10^{-4}$	607	901·7	$2·92\times10^{-3}$	$2·92\times10^{-4}$	675·0	718·3	$4·95\times10^{-3}$	$4·22\times10^{-4}$	340·1	1 228·1
9	$1·71\times10^{-3}$	$3·73\times10^{-4}$	394·5	889·6	$1·75\times10^{-3}$	$1·53\times10^{-4}$	456·1	748·4	$2·39\times10^{-3}$	$1·72\times10^{-4}$	284·0	1 182·2
10	$6·85\times10^{-3}$	$1·37\times10^{-4}$	316·1	873·1	$1·22\times10^{-3}$	$9·49\times10^{-5}$	347·2	749·0	$1·15\times10^{-3}$	$7·11\times10^{-5}$	216·9	1 091·8
11	$5·69\times10^{-4}$	$1·06\times10^{-4}$	246·1	843·0	$7·80\times10^{-4}$	$5·26\times10^{-5}$	266·2	691·6	$9·95\times10^{-4}$	$5·52\times10^{-5}$	176·3	1 020·4
12	$5·70\times10^{-4}$	$9·89\times10^{-5}$	173·2	764·9	$5·72\times10^{-4}$	$3·50\times10^{-5}$	206·7	632·9	$7·00\times10^{-4}$	$3·51\times10^{-5}$	138·1	916·1
13	$3·54\times10^{-4}$	$5·68\times10^{-5}$	154·2	660·0	$3·70\times10^{-4}$	$2·03\times10^{-5}$	181·6	583·6	$5·33\times10^{-4}$	$2·46\times10^{-5}$	123·5	810·1
14	$2·93\times10^{-4}$	$4·35\times10^{-5}$	127·4	590·9	$3·70\times10^{-4}$	$1·87\times10^{-5}$	142·3	540·3	$4·30\times10^{-4}$	$1·87\times10^{-5}$	108·0	725·9
15	$2·62\times10^{-4}$	$3·64\times10^{-5}$	93·5	525·1	$2·35\times10^{-4}$	$1·08\times10^{-5}$	122·6	490·6	$3·90\times10^{-4}$	$1·57\times10^{-5}$	94·0	636·5
16	$2·07\times10^{-4}$	$2·67\times10^{-5}$	74·2	424·4	$1·73\times10^{-4}$	$7·00\times10^{-6}$	95·6	426·7	$2·85\times10^{-4}$	$1·05\times10^{-5}$	80·5	548·5
17	$1·50\times10^{-4}$	$1·83\times10^{-5}$	59·8	371·3	$1·70\times10^{-4}$	$6·30\times10^{-6}$	71·9	366·5	$2·45\times10^{-4}$	$8·43\times10^{-5}$	67·8	477·5
18	$1·50\times10^{-4}$	$1·78\times10^{-5}$	54·2	302·9	$1·20\times10^{-4}$	$4·00\times10^{-6}$	62·2	308·3	$1·60\times10^{-4}$	$5·42\times10^{-6}$	61·6	404·9
19	$1·00\times10^{-4}$	$1·19\times10^{-5}$	49·0	237·0	$1·00\times10^{-4}$	$3·10\times10^{-6}$	49·0	250·1	$2·05\times10^{-4}$	$7·24\times10^{-6}$	54·2	344·9
20	$1·00\times10^{-4}$	$1·25\times10^{-5}$	44·9	185·9	$1·00\times10^{-4}$	$3·08\times10^{-6}$	40·7	211·5	$2·30\times10^{-4}$	$8·98\times10^{-6}$	47·8	298·4

[a] $\bar{M}_w$ (particle) $2·53\times10^{8}$ $\bar{M}_w$ (particle) $27·9\times10^{8}$ $\bar{M}_w$ (particle) $13·2\times10^{8}$.

The molecular weights $\bar{M}_w$ of the fractions 2 to 20 from Table 2 are plotted logarithmically against V_e in Fig. 6. They were used together with the intrinsic viscosities in Table 2 to establish the universal calibration line in Fig. 7. It is seen that, in fact, the three different pectins between 180 ml and 320 ml give a unique straight line of good quality verifying the two-component interpretation of the light-scattering data. Deviations from linearity close to the void volume are likely to be due to the accumulation effect of GPC in this region. The small but increasing deviations at V_e greater than 270 ml for Bulmer citrus pectin are assumed to be due to a delayed elution of this especially viscous pectin solution (Table 1) in agreement with general experience. However, with respect to subsequent light-scattering measurements, no reduction in concentration was possible to avoid this phenomenon.

The $[\eta]$ and $\bar{M}_w$ for each pectin give ultimately the same calibration line, but non-linear and different Mark-Houwink plots (Fig. 8). This

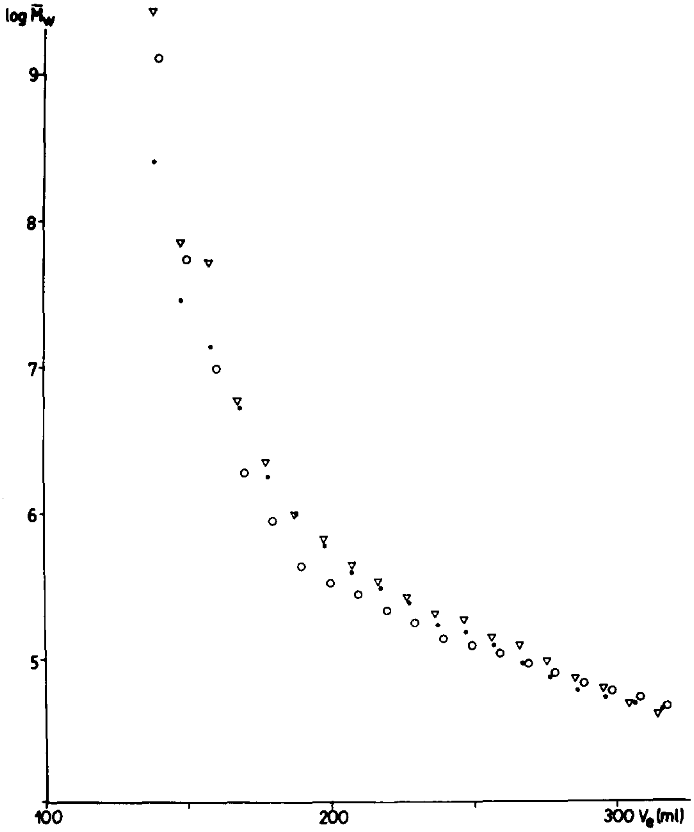

Fig. 6. Log $\bar{M}_w$ against the elution volume (for symbols see Fig. 2).

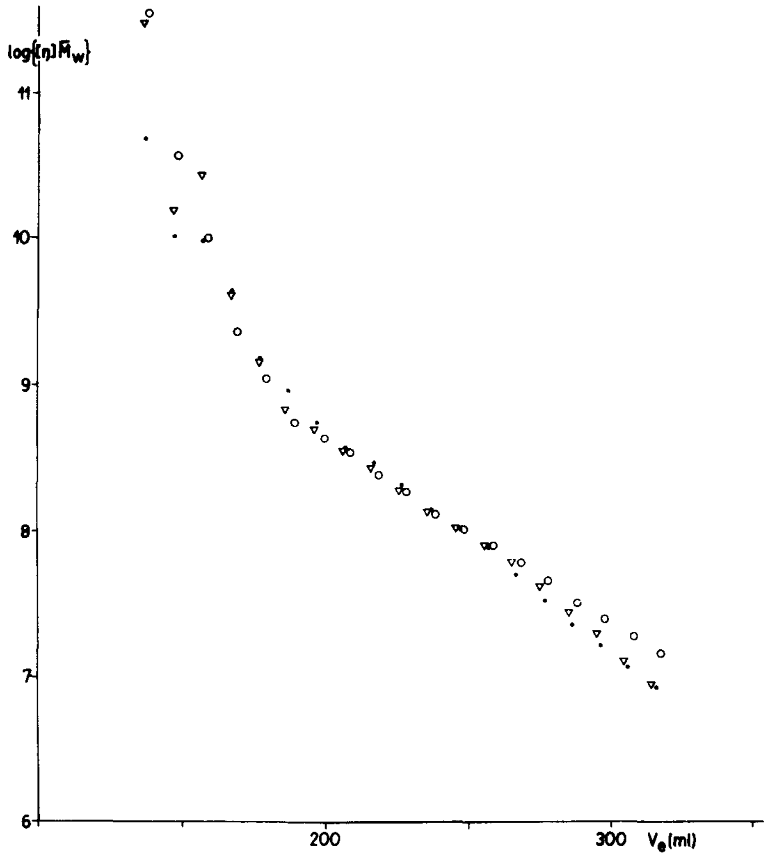

Fig. 7. Universal calibration line (for symbols see Fig. 2).

suggests that the non-particulate part of the GPC fractions is heterogeneous with a composition depending on pectin source. Heterogeneity was believed to be due to linear and branched molecules which are eluted together because they have the same hydrodynamic volumes (Berth, 1988). Branched molecules were detected by their neutral sugar side chains along the galacturonic acid polymer backbone. Consequently, different heterogeneities should be reflected in different neutral sugar–galacturonic acid ratios. This was proved by the E_q values from the UV spectra at 297 and 315 nm after the addition of sulphuric acid. E_q acquires values of 1·2 for pure galacturonic acid and 0·42 for glucose. Usually observed values for pectin GPC fractions are in the range 0·8 and 1·2.

The results obtained are shown in Fig. 9. E_q varies not only from fraction to fraction for a given pectin but also from one pectin to another

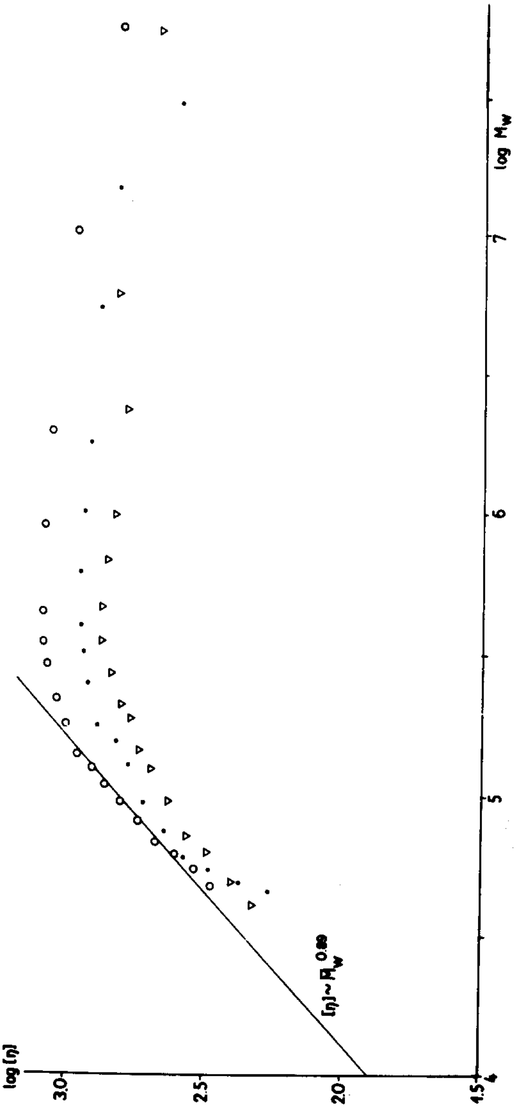

Fig. 8. Mark-Houwink plot (for symbols see Fig. 2).

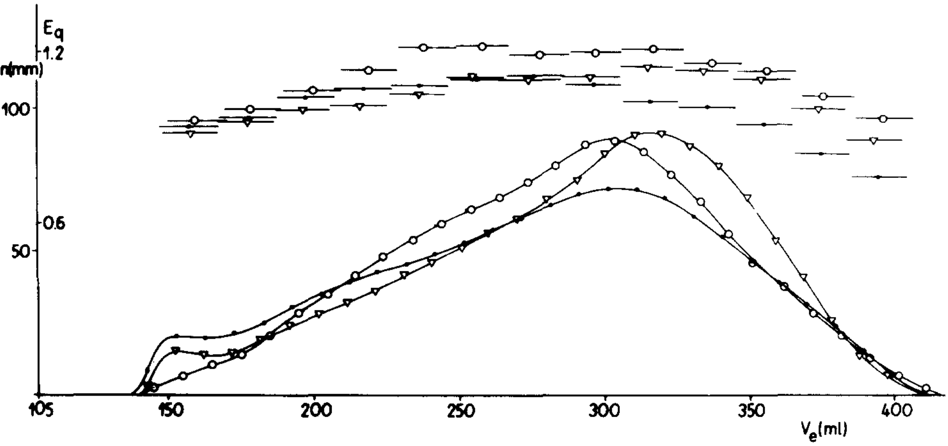

Fig. 9. Elution lines and E_q values for citrus pectin from Koch-Light, citrus pectin from Bulmer, and apple pectin type NSSU from Herbstreith (for symbols see Fig. 2).

at a given V_e. Comparing E_q and log$[\eta]$ (Fig. 4) we can observe as a rule that at any given V_e a small E_q value belongs to a low $[\eta]$ value and vice versa. This is consistent with the fact that branched molecules contribute less to the viscosity than linear molecules of the same molecular weight. These data cannot help to differentiate between an increasing degree of branching and an increased contribution of branched material in the fraction.

The neutral sugar distribution along the elution line can explain the level of the plateau and the different slopes of the log$[\eta]$–log $\bar{M}_w$ relationship in Fig. 8. Since Koch-Light citrus pectin was the same as used before (Berth, 1988) the separation achieved here was worse. This is indicated both by the lower E_q values around the peak maximum at GPC and therefore a decreased exponent $\alpha < 0{\cdot}89$. Only Bulmer citrus pectin was fractionated well enough to confirm $\alpha = 0{\cdot}89$ for pure galacturonic acid methyl ester polymers reported previously (Berth *et al.*, 1990).

Apple pectins regularly show some deviations from linearity on the side of decreasing molecular weights which are always higher than expected from the $[\eta]$ values. This is likely to be due to compact neutral sugar polymer fragments which were cleaved from the hairy regions during production and this is supported by other authors' results (Barbier & Thibault, 1982; Lecacheaux & Brigand, 1988) who found high galactose contents at the low-molecular end of the pectin elution.

Resuming, we are regularly faced with three pectin components characterised by their own size distributions and a preferred elution volume range. A complete separation by GPC cannot be achieved and perhaps no sharp borders do exist. To estimate their individual contributions, a range of techniques have been employed using the same sample of citrus pectin from Koch-Light Ltd, as a model (Berth, 1988; Berth *et al.*, 1989). Whereas our light-scattering interpretation in terms of a two-component system subdivides pectins into a particle component consisting of highly dense spheres on the one hand (about 5% for Koch-Light citrus pectin) and molecularly dispersed pectin (linear plus branched molecules) on the other hand, the derivation of heterogeneity from Mark-Houwink plots in connection with the universal calibration line and the elution profiles differentiates between linear and non-linear (branched molecules plus spheres). The non-linear component comprises about 15% of the Koch-Light citrus pectin). When the same approach was applied to Herbstreith apple pectin type NSSU, we obtained about 35% as non-linear contribution and 5–7% spheres.

CONCLUSIONS

These studies illustrate the influence of the heterogeneity of different pectins at GPC. Information on the molecular weight distribution of an unknown high-methoxyl pectin can be easily derived from GPC and simultaneous $[\eta]$ measurements if a well-defined universal calibration line is available. However, it should be kept in mind that all fractions consist of complex mixtures.

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