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Methodical aspects of characterization of alginate and pectate by light scattering and viscometry coupled with GPC

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The reliability of light scattering depends on the complete removal of extraneous matter from the polymer solution. The use of membrane filters to clarify aqueous solutions is common practice. This paper studies the effect of the pore size of the membrane filter on the light scattering of several polyuronides. Alginate and pectates were fractionated by GPC on Sepharose gels, and the eluent was taken after filtration for light scattering and viscosity measurements. Light scattering data were represented as GUINIER plots to provide information about the scattering functions and scattering level. While for alginate satisfactory separation of molecularly dispersed polymer from extraneous material was achievable, heterogeneity of pectates did not allow an unequivocal differentiation of extraneous matter from 'proper' pectate.

Dextran sulphate was included as a model substance which was free of extraneous material.

INTRODUCTION

Alginates as well as pectates derived from high-methoxyl commercial fruit pectins by enzymatic deesterification can be gelled with calcium ions to form beads for cell entrapment (Gemeiner *et al.*, 1989, in press). To correlate their functional properties with molecular parameters there is an interest in determining molecular weights or molecular weight distributions as well as other macromolecular features. Membrane osmometry, light scattering techniques and GPC coupled with light scattering detection are convenient tools for this purpose.

Unfortunately, the determination of molecular weights on polysaccharides is often subjected to disturbances by self-association and/or chemical and physical heterogeneity (Harding, Vårum *et al.*, 1991). Some effort and experience are needed to obtain reliable results, especially by light scattering, whether in combination with GPC or alone.

One of the critical points of light scattering experiments is the clarification of solutions. This means the removal of all extraneous matter such as 'dust, fibres and particles accumulated during polymer preparation' and, in the case of naturally occurring polymers, 'debris from other components of the material from which it was extracted' (Tabor, 1972). This is usually achieved by membrane filtration through filters of defined pore sizes making use of size differences between the solute of interest and extraneous material. It has become common practice to clarify polysaccharide solutions through membranes with pore sizes of 0·45 and 0·2 μm. Occasionally membrane filtration is combined with an ultracentrifugation step (Dingsøyr & Smidsrød, 1977). Having exhaustively purified the stock solution prior to analysis, the question arises whether results are still representative of the material originally dissolved.

Our experience with high-methoxyl pectins is fully consistent with that from other laboratories (Hourdet & Muller, 1987, 1991*a*, 1991*b*; Brigand *et al.*, 1990) and has shown both the need for a careful consideration of the clarification conditions and a sophisticated interpretation of the scattering functions in terms of either a two- or multi-component system (Berth *et al.*, 1990, 1991; Berth & Lexow, 1991). As the parent sample fractions were never narrowly distributed with respect to molecular weight or free from particulate matter, by studying solutions without any previous purification prior to GPC and analysing the scattering behaviour of the eluent as a function of the pore size of the membrane

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filters used for clarification (5·0 down to 0·1 μm), we have gained a better insight into the heterogeneity of the material. We had to learn that as long as there is no unequivocal criterion available for the discrimination of extraneous matter from the polymer being studied, all our clarification conditions or corrections by computation need to be related to some arbitrary standard and the results should be confirmed by other independent techniques as was done for pectins (Berth *et al.*, 1990; Harding, Berth *et al.*, 1991).

This report summarizes some experimental results obtained on a commercial alginate and two laboratory-made pectates (one from citrus and the other from apple pectin) by GPC coupled with light scattering and viscometry. Performing GPC on a semi-preparative scale on porous Sepharose gels, we were able to fractionate even slightly turbid solutions in order to observe the effect of clarification within the fractions with a stepwise decrease in pore size. In this way we can prevent the uncontrolled loss of strongly scattering matter during solution preparation and discuss in more detail what has happened to the solute.

We were aware of the lack of a method for assessing the total absence of small particulate nonuronide matter which makes it difficult to evaluate the success of these procedures. For this reason a 'model' polysaccharide with polyelectrolyte character was used in these studies to establish the universal calibration line for the GPC system and to assess the data obtained on polyuronides. Dextran sulphate was chosen as the 'model' material.

Some general comments concerning light scattering should be repeated. According to the basic formula

$$\frac{Kc}{R_\theta} = \frac{1}{\overline{M}_w} + \frac{1}{\overline{M}_w}\frac{(16\pi^2)}{(3\lambda^2)}\overline{\langle s^2\rangle}_z \sin^2\frac{\theta}{2} + 2A_2c + \ldots$$

the weight average molecular weight M_w is obtained from the intercept of the scattering curve at zero angle and zero concentration, while the initial slope of the angular dependent term represents a measure for the z-average of the radius of gyration $\langle s^2\rangle_z$ exp $\frac{1}{2}$. Both the shape of the scatterers and the polydispersity of the scattering system affect the angular dependent term (Kerker, 1969). Plotting the measured data as Guinier plot,

$$\ln\frac{R_\theta}{Kc} = \ln\overline{M}_w + \ln P(\theta)$$

has the great advantage that the scattering function ($P(\theta)$) is independent of all changes in the elution volume V_e and M_w can be immediately observed (Berth *et al.*, 1990). Thus by employing a Guinier plot the effect of clarification on the light scattering results can easily be discussed, at least qualitatively in terms of structural type and polydispersity of the scattering matter, since the intercept of the scattering curves with the ordinate ($\theta = 0°$) is equal to ln M_w neglecting the small concentration dependent contribution (A_2 term).

EXPERIMENTAL

The sodium alginate was a commercial sample (Fluka, FRG) and dextran sulphate was purchased from Pharmacia, Sweden. Preparation of the potassium pectates by enzymatic deesterification has been described elsewhere (Gemeiner *et al.*, 1989). Some data of these samples are listed in Table 1.

GPC on Sepharose C1-2B/Sepharose C1-4B was performed on two coupled columns (Pharmacia, Sweden; together about 380–400 ml) loaded with 30 mg polysaccharide/15 ml per run. 0·037 M phosphate buffer, pH 6·5, with the addition of 1 mmol/litre Na_2 EDTA was used as the solvent and eluent. Concentration of polymer in the eluent was monitored by means of a differential refractometer (Knauer, FRG). Fractions of ~10 ml were collected and subsequently studied by light scattering ('Sofica', Fica, France, equipped with a Zeiss helium-neon laser, Germany, of λ = 632 nm) and capillary viscosimetry ('Viscomatic', Fica, France).

Membrane filters were made by Sartorius-Membranfilter-GmbH (FRG). For each GPC run only one filter was used, and the fractions passed through it in the direction of decreasing fraction number. All excess scattering values R_θ are related to the blank eluent which had passed through the membrane first.

Number average molecular weights $\overline{M}_n$ were determined by membrane osmometry using a Knauer membrane osmometer, FRG, equipped with a membrane of pore size <5 nm (Sartorius-Membranfilter-GmbH, FRG). The composition of the solvent was the same as above with an addition of 0·02% sodium azide for conservation. Concentration series with four or five graduations between 1 and 5 mg/ml were employed. Further details are given elsewhere (Berth *et al.*, 1990).

RESULTS AND DISCUSSION

Calibration of the GPC system with dextran sulphate

Figure 1 shows the elution profile of dextran sulphate on the Sepharose columns. A good separation was achieved: polysaccharide elution covering the whole elution volume range between the void volume V_0 and the total volume V_t. The results of light scattering measurements on the fractions after filtration through a 0·45 μm pore size membrane filter are given as Zimm and Guinier plots in Fig. 2. The linear angular dependence shown in the Zimm plot is consistent with coil-like structures and gives no hint of the particularly broad molecular weight distributions in the fractions.

Table 1. Some chemical and physico-chemical data of the polyuronides used

Sample	η (ml/g)	$\overline{M}_n$[a]	Recovery (% of original)		Galacturonan content (% of original)	Yield of neutral sugars[b] (mg/100 mg original)				
			From GPC	From GC		Ara	Rha	Xyl	Gal	Glc
Alginate	462	59 750	90	—	—					
Citrus pectate	258	47 350	89	92	80	1·7	3·4	—	2·7	—
Apple pectate	247	54 750	90	89	60	1·3	3·2	0·4	0·2	17·3

[a]From membrane osmometry (Knauer instrument, FRG).
[b]As TMS derivatives after acidic methanolysis (GC/MS/DS 4515; Finnigan MAT, UK).

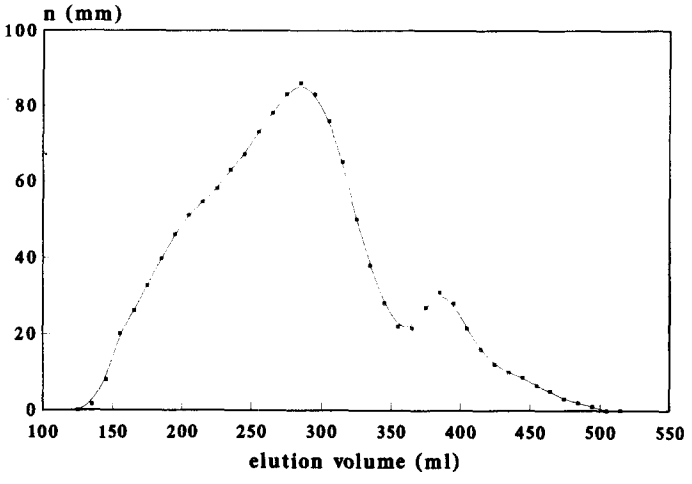

Fig. 1. Elution profile of dextran sulphate on Sepharose C1-2B/Sepharose C1-4B (points start with fraction number 1).

For small molecular weights ($M_w < 200\,000$) the angular dependence disappears.

As expected, the average molecular weight decreases with rising elution volume (Table 2) yielding together with the corresponding intrinsic viscosities (Table 2) a reliable basis for the universal calibration curve shown in Fig. 3.

When a membrane filter with 0·8 μm pore size instead of 0·45 μm is used, the scattering curves for identical fractions show quite another shape (Fig. 4). The strong curvature at low angles as well as the well-developed intensity minimum reveals the presence of large spherical particles in all fractions and, moreover, the surprising fact that the initial slope and hence the radius of gyration increases with increasing elution volume. As neither wide angle scattering intensities nor intrinsic viscosities depend on the pore size chosen for

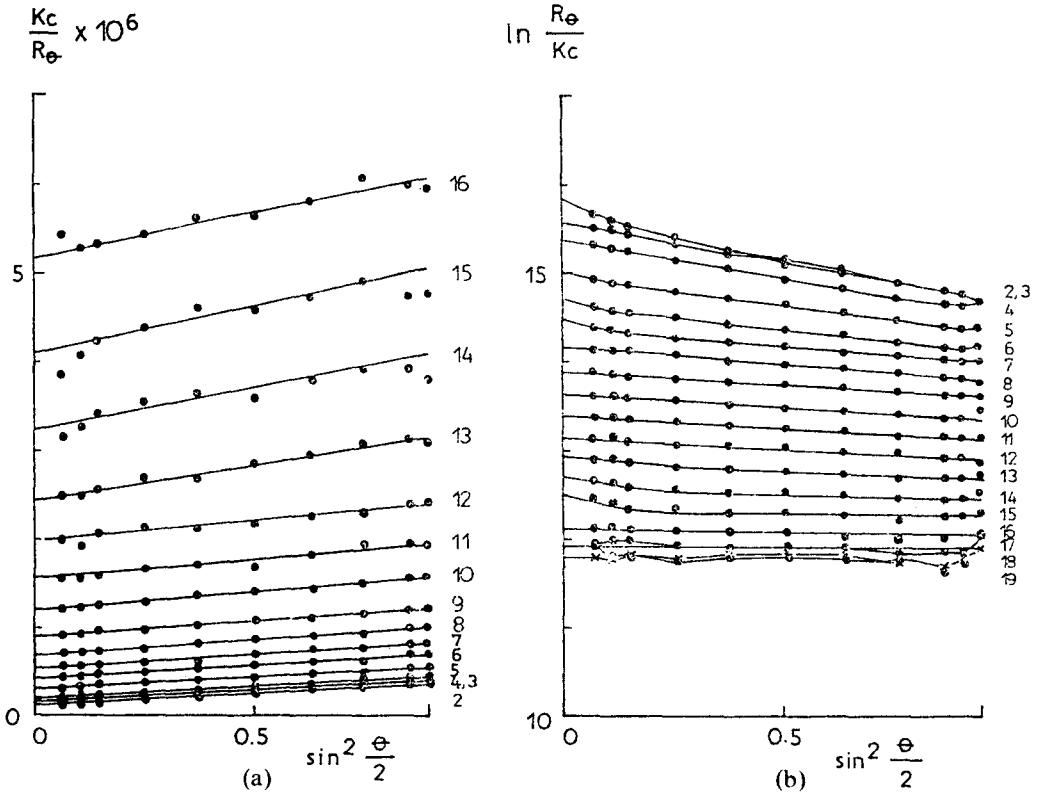

Fig. 2. Zimm (a) and Guinier (b) plot for the fractions according to Fig. 1 after filtration through 0·45 μm pore size membrane filter.

Table 2. Molecular weights $\bar{M}_n$ and intrinsic viscosities $|\eta|$ of the dextran sulphate fraction according to Fig. 1

Beginning of elution volume fraction (ml)	Number of fraction	Log $\bar{M}_w$	Log $\|\eta\|$
119·8	1	—	—
129·8	2	6·862	2·584
139·8	3	6·775	2·490
149·8	4	6·667	2·548
159·8	5	6·514	2·507
169·7	6	6·384	2·459
179·7	7	6·276	2·466
189·7	8	6·145	2·456
199·7	9	6·015	2·406
209·7	10	5·906	2·346
219·7	11	5·820	2·303
229·7	12	5·711	2·263
239·6	13	5·602	2·213
249·6	14	5·516	2·130
259·6	15	5·385	2·117
269·6	16	5·255	1·980
279·6	17	5·190	1·928
289·6	18	5·125	1·857
299·6	19	5·103	1·997

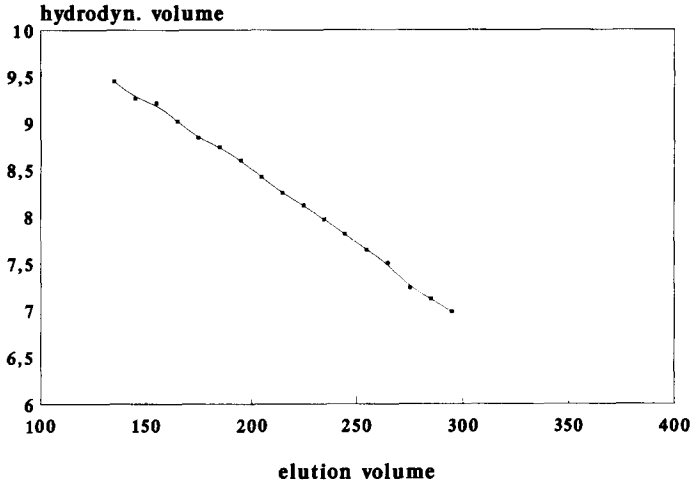

Fig. 3. Universal calibration plot for the GPC system established with dextran sulphate.

clarification, differences between Fig. 2(b) and Fig. 4 are obviously due to small amounts of large 'extraneous' particulate matter which can easily be removed from the proper solute (the molecularly dispersed dextran sulphate) by membrane filtration. This represents a simple example of the aim of clarification and makes a quantitative interpretation of Fig. 4 unnecessary and confirms the validity of the relationship shown in Fig. 3.

The average molecular weight for the measurable region from fraction 2 to 20 was found to be approximately $M_w \sim 740\,000$ neglecting the 'low molecular weight' tail ($M_w < 100\,000$).

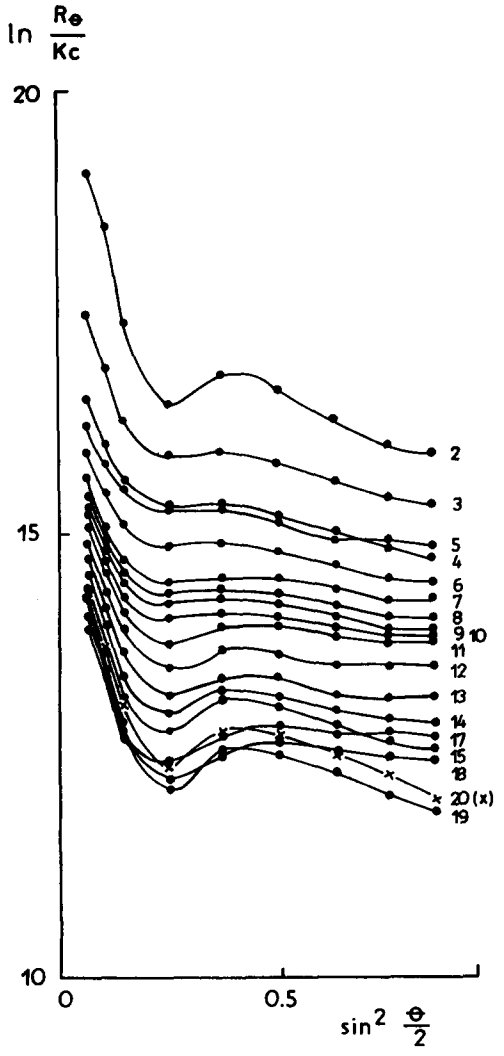

Fig. 4. Guinier plot for the fractions according to Fig. 1 after filtration through 0·8 μm pore size membrane filter.

Studies on alginate

The elution profile of alginate (Fig. 5) indicates a broad size distribution beginning with a slight accumulation peak at V_0 and ending with a shoulder on the main peak in the middle of the elution range.

Guinier plots in Fig. 6(a)–(c) illustrate the strong influence of clarification conditions on the light scattering results. Although no detectable loss in concentration occurs, both reduction in absolute scattering level and altered shapes of scattering functions suggest a decrease in the mass contribution and particle diameter when the pore size was diminished stepwise. (It is fair to mention that Fig. 6(a) comes from a separate GPC run while Fig. 6(b) and (c) represent the results of a successive diminution in pore size. In the case of 0·2 μm pore size the flow rate was reduced and the 0·1 μm filter blocked.)

To achieve similarly simple scattering curves as were found for dextran sulphate fractions, smaller pore sizes

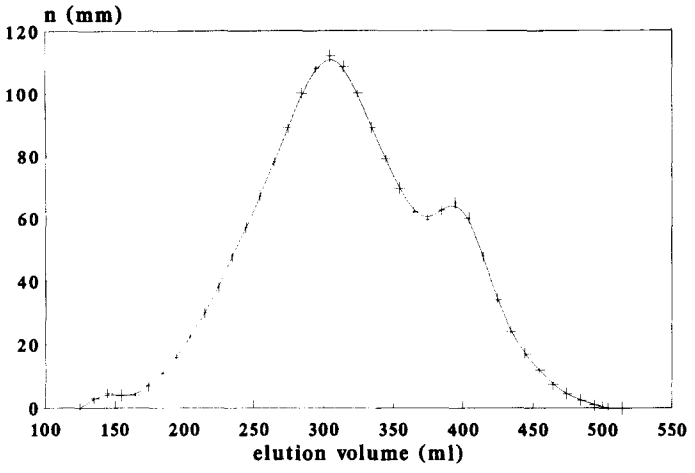

Fig. 5. Elution profile of alginate on Sepharose C1-2B/ Sepharose C1-4B (points start with fraction number 1).

are necessary, then excess scattering intensities become so small that they can hardly be measured with the precision needed for exact molecular weight determinations.

Nevertheless, if we neglect low angles and take the more accurately measurable data out of the wide angle region to calculate $\overline{M}_w$, the values obtained have a negligible error.

Comparing now the molecular weight level for corresponding fractions from alginate and dextran sulphate we find what was expected from our traditional understanding of size exclusion chromatography: for a given hydrodynamic volume or V_e the more compact dextran sulphate molecules possess a higher average molecular mass than the more extended stiff polyuronide.

Figure 7 shows that filtration has no significant effect on the intrinsic viscosities. Thus it can be concluded that filtration has removed exclusively high molecular weight particulate material, as was also concluded by Mackie & Noy (1980) from studies on unfractionated samples, but has left all the molecular dispersed alginate in solution. However, the presence of very small residual particles giving no effect on the angular dependence of the light scattering intensity but increasing the 'background' level, cannot be definitely excluded at this stage. They should reveal themselves by 'positive' deviations of the actual from the well established universal calibration line (Fig. 3). This is what is indeed obtained. Figure 8 shows that the alginate data deviate from the dextran sulphate universal calibration line. The deviation which rises with the elution volume might also be due to a delay in eluting the highly viscous alginate solution, and further

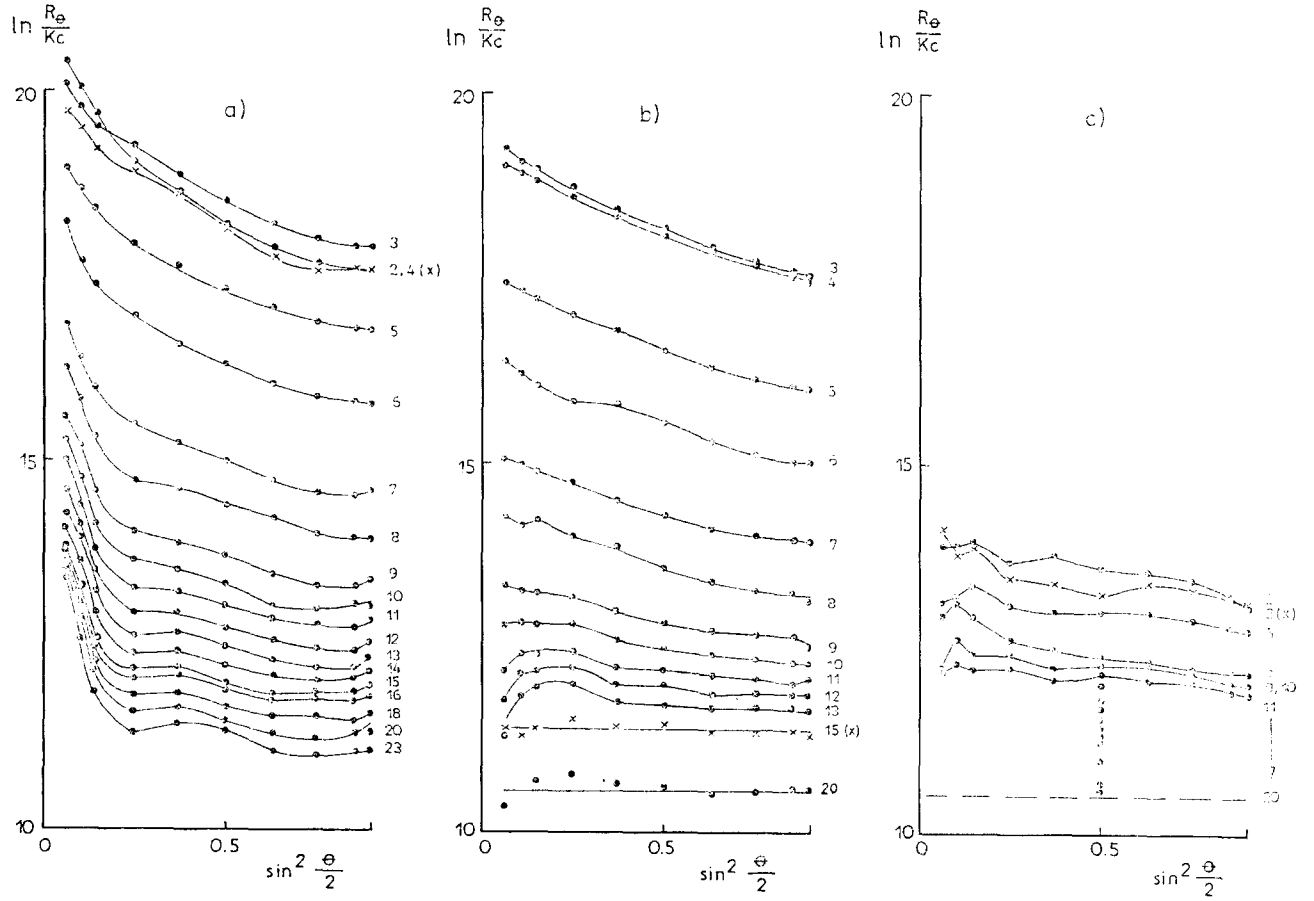

Fig. 6. Guinier plots for the fractions according to Fig. 5 after filtration through (a) 0·8, (b) 0·45 and (c) 0·2 μm pore size membrane filters.

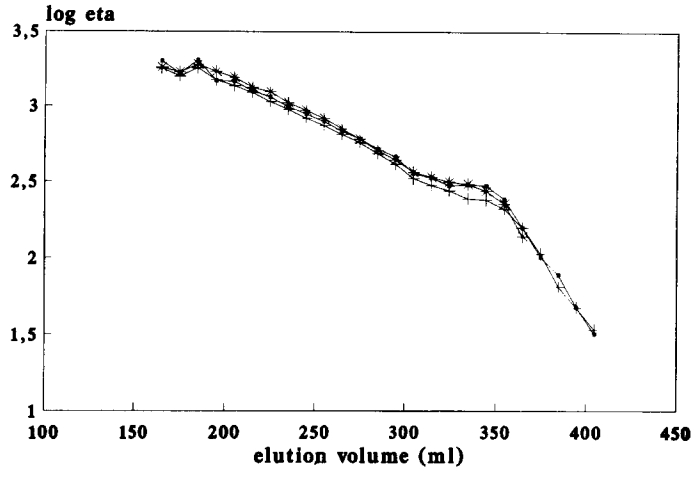

Fig. 7. Logarithmic intrinsic viscosities against the elution volume for the fractions according to Figs 5 and 6 (□ — 0·8 μm; + — 0·45 μm; * — 0·2 μm).

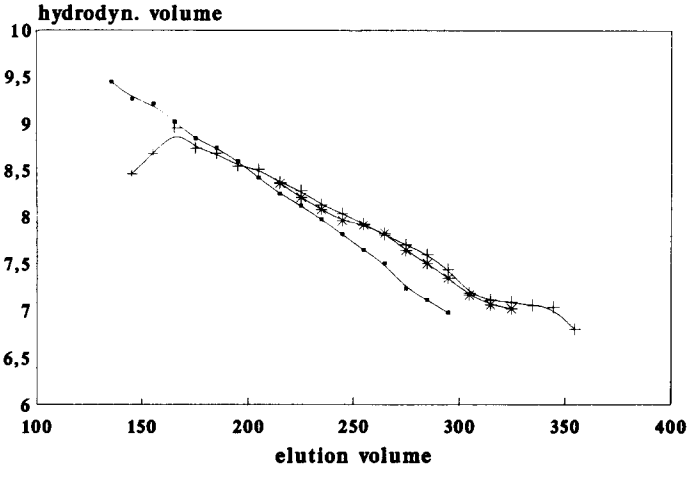

Fig. 8. Universal calibration plot for alginate on the basis of data in Figs 6(c) and 7 (+) or calculated M_w data (*) in comparison to Fig. 3.

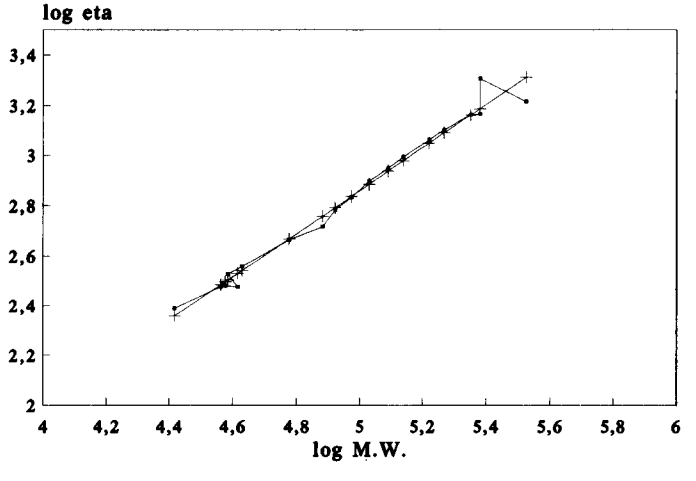

Fig. 9. Mark-Houwink plot for alginate (0·2 μm); (+) — linear regression line.

experiments are needed to clarify this. It is worth mentioning that the same 'reduction' in molecular weights obtained experimentally by light scattering of solutions filtered through small pore sizes could be achieved by computation applying formally the two-component algorithm for the interpretation of the angular dependence originally developed for a special type of scattering curve series (Berth *et al.*, 1990; Berth & Lexow, 1991). Based on the data in Fig. 6(b) calculations lead to a plot very similar to Fig. 6(c).

Plotting the intrinsic viscosities against molecular weights on a logarithmic scale, the strictly linear Mark-Houwink plot in Fig. 9 is obtained where the exponent α acquires a value close to 0·9. This is still consistent with data recently published by Smidsrød's group (Martinsen *et al.*, 1991) albeit both $\overline{M}_w \sim 82\,000$ and $\overline{M}_n \sim 60\,000$ were found to be a little lower for the Fluka product used here. In full agreement with that group (see also Strand *et al.*, 1982) we consider filtration through 0·2 μm pore size membranes, possibly combined with an ultracentrifugation treatment, as an adequate route for clarification of alginate solutions in order to remove sphere-like material as was already proven by electron microscopy (Smidsrød & Haug, 1968).

Up to here there were no hints for additional methodical problems caused by copolymeric nature of alginates.

Studies on pectates

The elution lines for the two pectates (Fig. 10) show great similarity to those of high-methoxyl pectins (Berth & Lexow, 1991) and so does the dependence of their light scattering behaviour on filtration conditions (Figs 11 and 12) (Kravtchenko *et al.*, 1992).

Because of the shortage of material the effect of 0·2 μm pore size membranes could not be tested, but

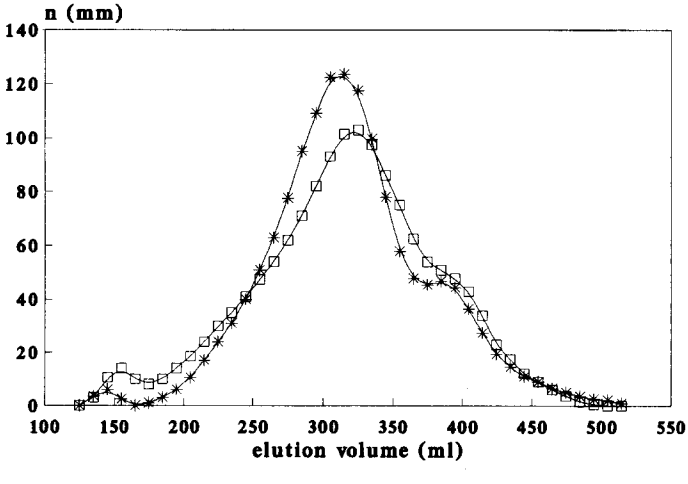

Fig. 10. Elution profiles of citrus (*) and apple (□) pectate on Sepharose C1-2B/Sepharose C1-4B (points start with fraction number 1).

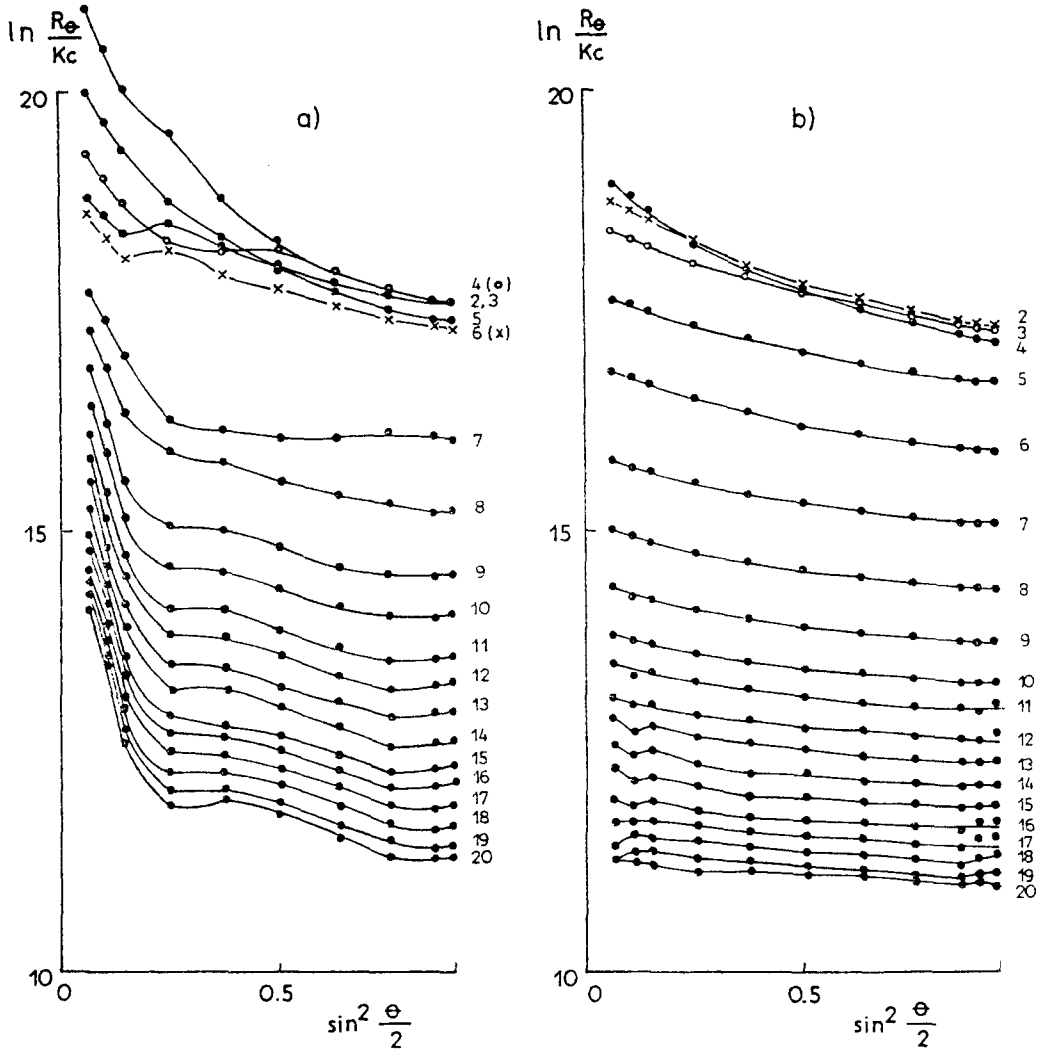

Fig. 11. Guinier plot for apple pectate fractions according to Fig. 10 after filtration through (a) 0·8 and (b) 0·45 μm pore size membranes.

from previous experience on high-methoxyl pectins it is reasonable to assume filtration through small pore size filters is linked with a loss in pectinaceous matter which makes a non-negligible viscosity contribution. For this reason we analysed the data by the computation method previously described (Berth & Lexow, 1991). This was shown to lead to good agreement with the universal calibration line for alginate shown in Fig. 8 in the case of citrus pectate and also for apple pectate when it was ultracentrifuged to remove even small dense particles. The corresponding Mark-Houwink plots for pectate shows the non-proportionality between $\log/\eta/$ and $\log \bar{M}_w$ for the high molecular weight region just as their high-methoxyl analogues (Berth, 1988). For the low molecular weight region ($M_w < 100\,000$) the exponent α is found to be approximately 1·2, supporting the rod-like conformation of polyuronides (Axelos, 1990; Harding, Berth *et al.*, 1991).

Because of the extreme heterogeneity of pectates (pectins) which is shown by the complex Mark-Houwink relationship and light scattering and GPC behaviour, there was no convincing criterion for differentiation between 'extraneous' matter (cell wall debris?) and 'proper' pectate (pectin) which might provide a basis for general recommendations as was possible in the case of alginate. This may explain the contradicting and confusing conclusions in the literature about pectins/pectates.

CONCLUSIONS

GPC is often overestimated for what it can afford in fractionating very polydisperse, heterogeneous materials. Effects described above play a less important role if samples to be studied have been intensively 'prepurified', for example when high-tech HP-SEC columns are used which must be protected against blocking.

A lot of information on the success of sample preparation and/or fractionation is 'hidden' in the

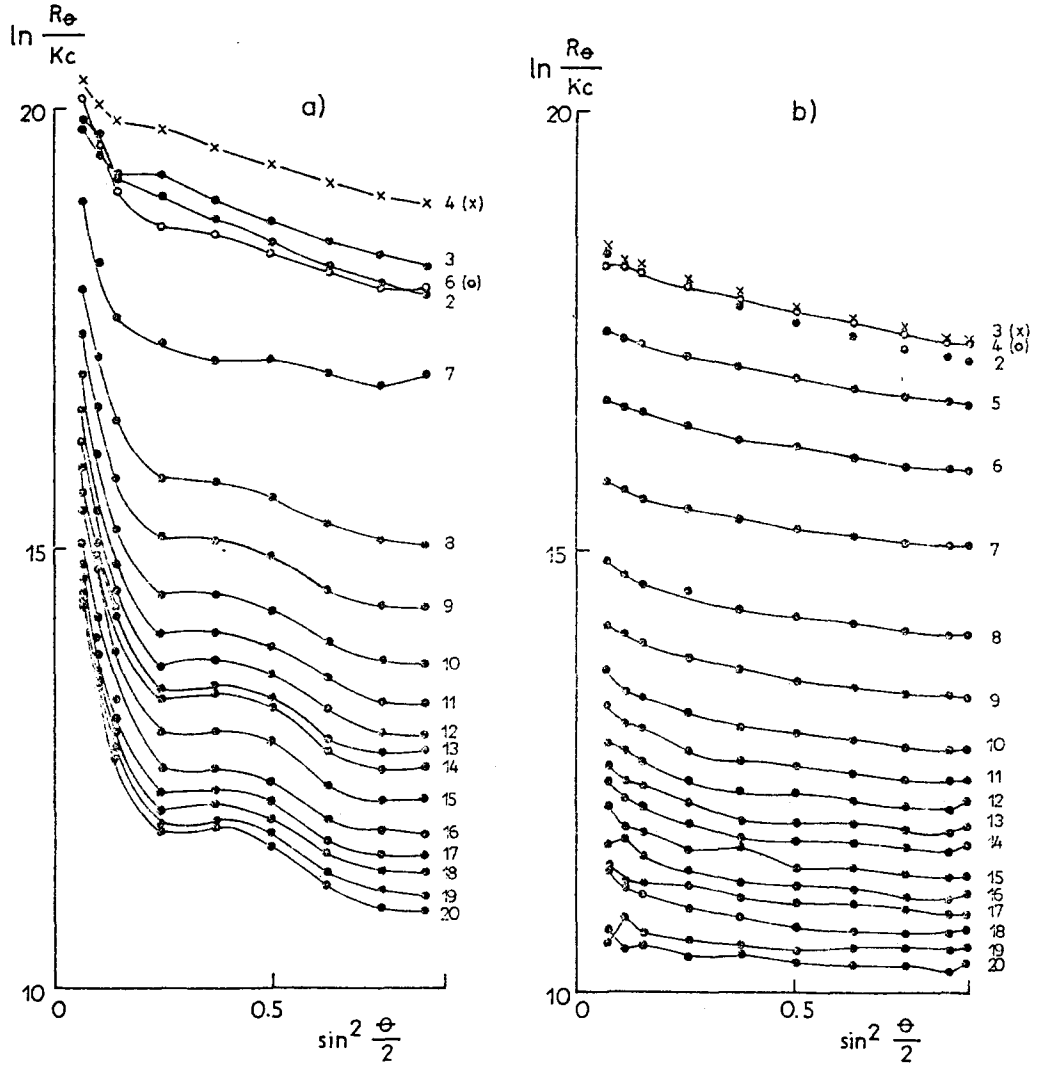

Fig. 12. Guinier plot for citrus pectate fractions according to Fig. 10 after filtration through (a) 0·8 and (b) 0·45 μm pore size membranes.

scattering functions of the eluent slices. At present multiangle-laser-light scattering (MALLS) detectors are to be preferred to low-angle-laser-light scattering (LALLS) equipment. Some quantitative improvements are still achievable in routine checking the experimental curves on the basis of model calculations, for example as was proposed by Dautzenberg & Rother (1988) and performed for high-methoxyl pectin (Berth *et al.*, 1991).

It seems too optimistic to expect good agreement in calibrations from different heterogeneous polymers. Staverman's view (1983) that the universal calibration works on the basis of $\overline{M}_n$ values has yet to be proven.

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REFERENCES

Axelos, M.A.V. (1990). *Makromol. Chem.*, **39**, 323–8.
Berth, G. & Lexow, D. (1991). *Carbohydr. Polym.*, **15**, 51–66.
Berth, G., Dautzenberg, H., Lexow, D. & Rother, G. (1990). *Carbohydr. Polym.*, **15**, 39–59.
Berth, G., Dautzenberg, H. & Rother, G. (1991). 6th Conference on Gums and Stabilizers for the Food Industry, Wrexham, UK.
Brigand, G., Denis, H., Grall, M. & Lecacheux, D. (1990). *Carbohydr. Polym.*, **12**, 61–77.
Dautzenberg, H. & Rother, G. (1988). *J. Polym. Sci., Part B, Polymer Physics*, **26**, 353–66.
Dingsøyr, E. & Smidsrød, O. (1977). *Brit. Polym. J.*, **9**, 56.
Gemeiner, P., Kurillova, L., Malovikova, A., Toth, D. & Tomasovicova, D. (1989). *Folia Microbiol.*, **34**, 214–27.

Gemeiner, P., Kurillova, L., Markovic, O., Malovikova, A., Uhrin, D., Ilavsky, M., Stefuca, V., Polakovic, M. & Bales, V. (1991). *Biotechn. & Appl. Biochem.*, **13**, 335–45.

Harding, S.E., Vårum, K.M., Stokke, B.T. & Smidsrød, O., (1991). In *Advances in Carbohydrate Analysis, Vol. 1*, ed. C.A. White. JAI Press Ltd, London & Greenwich, Connecticut, pp. 63–144.

Harding, S.E., Berth, G., Ball, A., Mitchell, J.R. & Garcia de la Torre, J. (1991). *Carbohydr. Polym.*, **16**, 1–15.

Hourdet, D. & Muller, G. (1987). *Carbohydr. Polym.*, **7**, 301–12.

Hourdet, D. & Muller, G. (1991*a*). *Carbohydr. Polym.*, **16**, 113–35.

Hourdet, D. & Muller, G. (1991*b*). *Carbohydr. Polym.*, **16**, 409–32.

Kerker, M. (1969). *The Scattering of Light and Other Electromagnetic Radiation.* Academic Press, New York, San Francisco, London.

Kravtchenko, T.P., Berth, G., Voragen, A.G.J. & Pilnik, W. (1992). *Carbohydr. Polym.*, **18**, 253–63.

Mackie, W. & Noy, R. (1980). *Biopolymers*, **19**, 1839–60.

Martinsen, A., Skjåk-Braek, Smidsrød, O., Zanetti, F. & Paoletti, S. (1991). *Carbohydr. Polym.*, **15**, 171–93.

Smidsrød, O. & Haug, A. (1968). *Acta Chem. Scand.*, **22**, 797.

Staverman, A.J. (1983). *Eur. Polym. J.*, **19**, 973–7.

Strand, R.A., Boe, A., Dalberg, P.S., Sikkeland, T. & Smidsrød, O. (1982). *Macromolecules*, **15**, 570–79.

Tabor, B.E. (1972). In *Light Scattering from Polymer Solutions*, ed. M.B. Huglin, Academic Press, London, New York, pp. 1–25.