Carbohydrate Polymers **22** (1993) 87–92

On the characterization of polygalacturonate salts in dilute solution

Anna Malovikova

Institute of Chemistry, Slovak Academy of Sciences 84238 Bratislava, Czech Republic

Marguerite Rinaudo & Michel Milas

Centre de Recherches sur les Macromolécules Végétales, CNRS, affiliated with the Joseph Fourier University of Grenoble, B.P. 53 X, F-38041 Grenoble cedex, France

(Received 29 September 1992; revised revision received 2 November 1992; accepted 20 January 1993)

Three de-esterified pectins have been studied by size exclusion chromatography (SEC) coupled with a multiangle laser light scattering detector. The molecular weight and radius of gyration distributions were established. The influence of the pore diameters of the membrane used to clarify the pectin solutions before SEC analysis was tested. Filtration through membranes 0·05 μm pore diameter removes high molecular weight material for which the measured radius of gyration is near to or higher than 0·04 μm. The origin of these large particles which are more compact and perhaps less sensitive to shear forces than the other fractions, was not investigated. Nevertheless, some high molecular weight matter cannot always be removed by filtration. As a consequence, the $\overline{M}_w$ of the main fraction of the sample, excluding the high molecular weight matter, is usually low and represents roughly 85% of the initial sample weight. The use of a refractive index detector coupled with SEC, or the measurement of the average molecular weight by light scattering cannot always demonstrate the presence of a high molecular weight fraction in pectin solutions.

INTRODUCTION

The characterization of both the shape and molecular weight is very important in understanding the physicochemical properties of pectins, but, the reported literature data suggest that these parameters are not being satisfactorily determined. For example, the $[\eta]$-M relationship varies depending upon the authors (Hourdet & Muller, 1991*a*). These differences may be due to heterogeneity of the primary structure, but can also be caused by the presence of aggregates or a high molecular weight fraction of the material which strongly affect the light scattering data particularly at low angles and here the measured molecular weight, whereas the viscosity is only slightly modified. The work of Hourdet & Muller (1991*b*) suggested that the true molecular weight of pectin could be determined by low-angle laser light scattering (LALLS) only after complete removal of high molecular weight substances. Another way to overcome such problems is the use of size exclusion chromatography (SEC). Recently, Anger and Berth (1985, 1986), Rombouts & Thibault (1986), Hourdet & Muller (1987, 1991*a,b*) Brigand *et al.* (1990), and Kravtchenko *et al.* (1992*a*) have used this technique to fractionate and analyse pectins. These studies gave information about the heterogeneity in the molecular size of pectin molecules and the chemical composition in relation to the molecular size. The recent and detailed analysis of SEC fractions of different pectins by Kravtchenko *et al.* (1992*a*) exhibit quite a complex situation because pectin molecules from different preparations, with the same molecular weight, can have different hydrodynamic volumes due to differences in degree of methoxylation, of branching and of neutral sugar content (Michel *et al.*, 1982; Kravtchenko *et al.* 1992*b*, *c*).

The high molecular weight matter may consist of aggregated molecules because of, for example, the strong tendency of pectins with low degrees of methoxylation to form aggregates in the presence of calcium

Carbohydrate Polymers 0144-8617/93/$06.00

ions. Other mechanisms of aggregation such as hydrogen bonds or hydrophobic interactions may occur in the absence of calcium ions (Davis *et al.*, 1980). New information has been obtained using SEC coupled with light scattering and/or viscosity measurements (Hourdet & Muller, 1991*a*,*b*); Berth, 1992; Kravtchenko *et al.* 1992*a*). These techniques clearly indicate that a fraction with a given hydrodynamic volume remains highly heterogeneous on the basis of chemical composition, conformation and molecular weight. In addition, it has been shown from light-scattering measurements not directly taken on line with SEC (Hourdet & Muller, 1991*a*, *b*; Berth, 1992; Kravtchenko *et al.*, 1992*a*) that it might also be possible that some of the high molecular weight matter is formed after SEC fractionation. These aggregates may have been disrupted during SEC fractionation by shear forces and reformed on resting before light-scattering measurements are taken. It is clear that the nature of this high molecular weight component has not yet definitively been elucidated.

In this paper we describe the molecular weight and radius of gyration distributions obtained on line by SEC for three pectins from different sources, de-esterified by different processes and with varying galacturonic acid contents. The goal of these characterizations is to determine the molecular weight of the main pectin fraction which can then be related to the hydrodynamic parameters and physicochemical properties of the polygalacturonate chains without the complicating effect of the high molecular weight component. No relation between the chemical composition (neutral sugar content) and the molecular weight distribution or high molecular weight material has been investigated.

EXPERIMENTAL

Different pectates were used. Sample 1 was the sodium salt of polygalacturonic acid from Sigma; sample 2 was potassium pectate prepared by de-esterification of commercial citrus pectin (Genu Pectin, Medium Rapid Set, Type A, Pektinfabrik, Copenhagen, Denmark) with enzymes (Gemeiner *et al.*, 1991) and sample 3 was potassium pectate prepared by de-esterification of commercial apple pectin (East Bohemian Canning Factories and Distilleries, Smirice, Czechoslovakia) with alkali (Tibenský & Kohn, 1970).

Samples 1 and 2 were purified by precipitation with 60% isopropanol or ethanol in the presence of NaCl (0·3–0·5 M). In the case of sample 3 commercial apple pectin was washed prior to de-esterification with hydrochloric acid acidified ethanol (5 ml of concentrated HCl in 100 ml of 60% ethanol) then with neutral 60% and 96% ethanol.

The content of galacturonic acid (Gal A) was determined by the precipitation of copper pectates and pectinates (Tibenský *et al.*, 1963; Kohn & Tibenský, 1965) and by potentiometry (Ravanat, 1979); microanalysis was performed to determin the Ca^{2+} content (Table 1). Gel permeation chromatography was carried out on our equipment fitted with a range of detectors as previously described (Tinland *et al.*, 1988). The eluent was 0·1 M NH_4NO_3 with 0·5 g/litre NaN_3 as preservative. The concentration of pectate solutions for SEC was 3 g/litre. Two columns, Shodex OH-pak 804 and 805, were used in series. The value of d*n*/d*c* was determined in the same solvent as 0·155 ml/g with a differential refractometer (Brice-Phoenix).

The solutions were filtered on Sartorius membranes before injection on the chromatograph; two porosities were used successively: 0·2 μm and 0·05 μm.

The light-scattering detector is a multiangle DAWN, model F, laser photometer equipped with the ambient flow cell for aqueous solution, K5, and the software ASTRA1-1 (Wyatt Technology Corporation, USA). Normalization was achieved from an NaPSS standard $M_w = 19\,300, M_w/M_n < 1{\cdot}10$). This equipment allows the simultaneous measurement of light scattered intensity for fourteen angles between 21 and 150° during elution. An example of a Zimm plot obtained for different elution volumes is shown in Fig. 1. The assumption that the maximum concentration (less than 0·1 g/l at the top of the refractive index signal) is low enough to neglect extrapolation to zero concentration was made. The molecular weight and the radius of gyration were estimated on different points of the chromatograms as shown on the individual figures. The corresponding elution volume, molecular weight and radius of gyration ($(\langle\rho^2\rangle^{1/2})$ are given in Table 2. Reproducible gyration radii were obtained for different polysaccharides for $(\langle\rho^2\rangle^{1/2}) > 130$ Å. Figure 2 shows the log–log plot of $\langle\rho^2\rangle^{1/2}$ *versus* M obtained from SEC for the sample 2. The characteristic slope 0·57 corresponds to that which can be predicted from the theoretical model of Odijk and Houwaart (1978). These results allow us to have confidence in $\langle\rho^2\rangle^{1/2}$ values higher than 130 Å. From the refractometric area signal the ASTRA1-1 software allows the determination of the

Table 1. Properties and composition of the different samples used

Samples	[η] NaCl 0·1 M (ml/g)	% Gal A	Ca^{2+} content (mol/g)	w(%) retention on 0·2 μm filter	w(%) retention on 0·05 μm filter
1	65·5	95	$4{\cdot}4 \times 10^{-6}$	1·2	0
2	211	87·4	$1{\cdot}5 \times 10^{-5}$	0·16	5·4
3	104	78·8	0	—	3

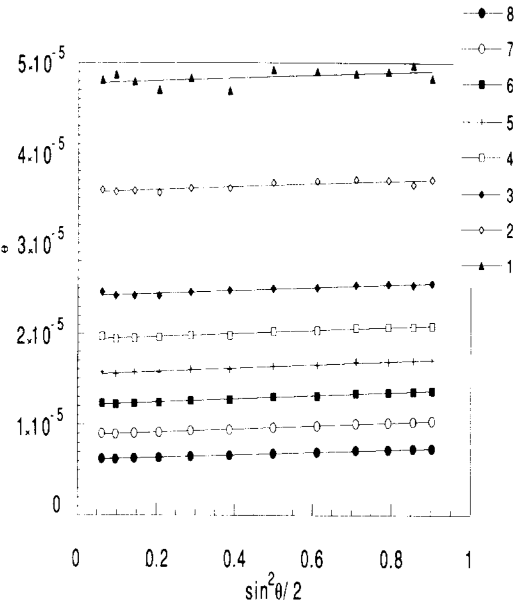

Fig. 1. Zimm plot, $Kc/\Delta R_\theta$ *versus* $\sin^2\frac{\theta}{2}$, at different slices (see Table 2) for a SEC experiment on sample 2 after filtration through 0·2 μm.

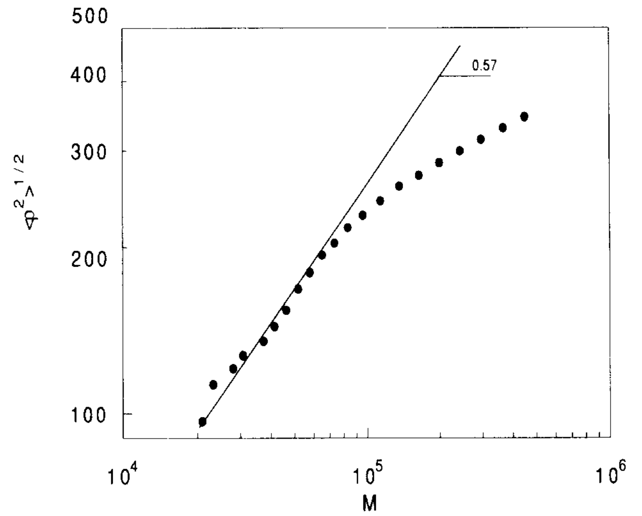

Fig. 2. Log–log plot of the radius of gyration $\langle \rho^2\rangle^{1/2}$ *versus* M obtained from a SEC experiment on sample 2 after filtration through 0·2 μm.

eluated polygalacturonate mass and hence the mass retained on filters during the SEC experiment ($dn/dc = 0{\cdot}155$ ml/g). The weight-average molecular weight was also determined.

RESULTS AND DISCUSSION

The chromatograms and the solution concentrations may be modified by the porosity of the membranes used for filtration, depending upon the average dimensions of the high molecular weight matter. Some may be larger than 0·2 μm and thus retained before elution. With the systems tested in this work, less than 1·2% of materials was retained on 0·2 μm membranes. These percentages were calculated from the determination of carboxyl groups before and after filtration by the pectate precipitation method and were confirmed from the weight of freeze-dried matter before and after filtration of 1 g/litre polygalacturonate solutions.

The weight fraction of high molecular weight material with dimensions between 0·2 and 0·50 μm was estimated from the difference in the area of the refractometric signals obtained for samples filtered on 0·2 μm and 0·05 μm. The results are given in Table 1. No relation exists between the loss on 0·2 μm and 0·05 μm filters.

The chromatograms obtained were compared on the different figures. As a first hypothesis, the chromatograms were decomposed into one, two or three parts using the light-scattering signals at a scattered angle of 31° and the refractometric signals as shown as shown in Fig. 3. To estimate the weight-average molecular weight $\overline{M}_{w_i}$ of the weight fraction (w_i) of each species, the area of these decomposed signals has been used. We have verified that the total $\overline{M}_w$ corresponds to the additivity of the individual contributions $\overline{M}_w = \Sigma w_i M_{w_i}$.

The results of the peak decomposition are given in Table 3. The chromatograms of sample 1 (Fig. 4) demonstrate that there is no change in the molecular weight distribution after filtration 0·2 and 0·05 μm. This means that the high molecular weight material causing the bimodal curves has dimensions lower than about 0·05 μm. This assumption is confirmed from the values of the radius of gyration ($\langle \rho^2\rangle^{1/2}$) given at various points on the chromatogram. They are always lower than the pore diameters of the filters. Addition of $CaCl_2$ in concentration corresponding to $4 \cdot 10^{-5}$ moles of Ca^{2+} per gram of polygalacturonate has not brought about any change in the chromatograms, which means that the high molecular weight matter which pre-exists in the purified polymer seems not to be due to Ca^{2+} ions but to large and dense particles which might be either branched pectin molecules or relatively stable aggregates.

Table 2. Molecular weight and radius of gyration *versus* elution volume for sample 2 taken from ASTRA 1-1 software and the data in Fig. 1.

Slice	1	2	3	4	5	6	7	8
V_e (ml)	30·24	29·09	27·70	27·00	26·31	25·62	24·92	24·22
M	20 960	28 140	41 600	52 030	65 280	83 666	114 038	165 000
$\langle \rho^2\rangle^{1/2}$ (Å)	96	120	143	167	193	216	243	270

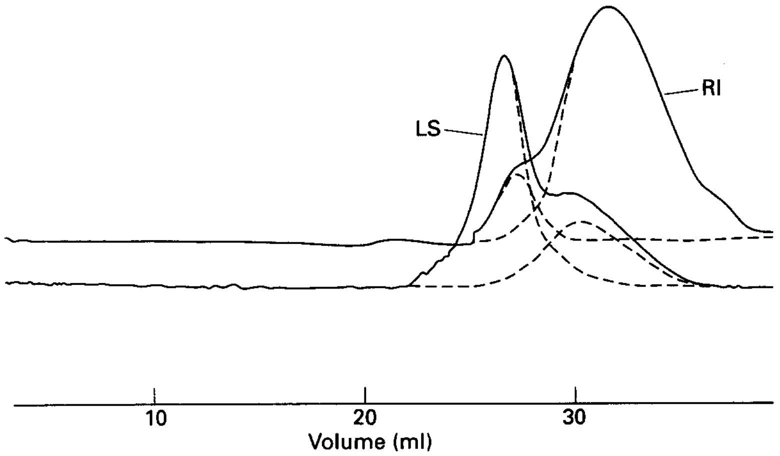

Fig. 3. Example of chromatogram decomposition for sample 3 after filtration through 0·05 μm (RI, refractive index; LS, light scattering).

Table 3. Average molecular weights (M_{w_i}) and weight fractions (w_i) for the different parts of the chromatogram decomposition obtained after filtration through 0·2 and 0·05 μm compared to the average molecular weight of the samples ($\overline{M}_w$)

Samples	Filtration (μm)	M_{w_i}			w_i(%)			$\overline{M}_w$
		1	2	3	1	2	3	
1	0·2	—	137 500	23 500	—	15	85	40 600
	0·05	—	138 400	24 000	—	15	85	41 200
2	0·2	—	570 000	83 800	—	5·4	94·6	109 960
	0·05	—	—	83 820	—	—	100	83 800
3	0·2	$1{\cdot}510^{6}$	650 000	35 000	3	12	85	153 000
	0·05	—	269 500	34 800	—	15	85	70 000

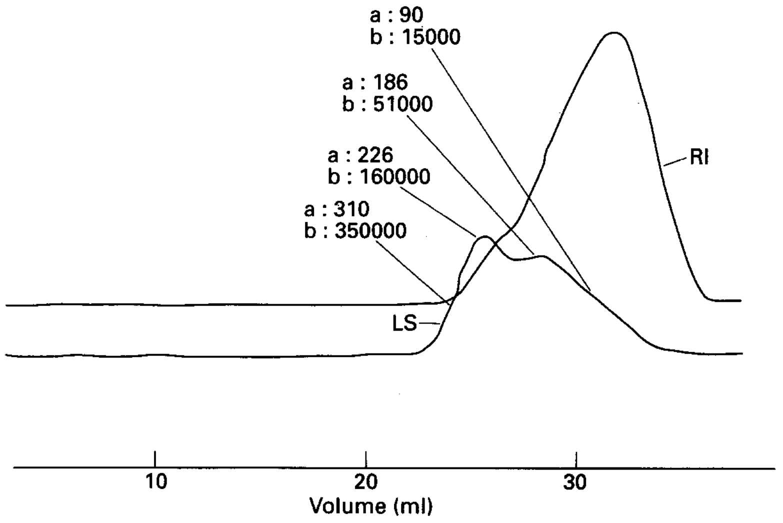

Fig. 4. Chromatograms of sample 1 after successive filtrations through 0·2 and 0·05 μm, where a = radius of gyration (Å), b = molecular weight at the indicated positions (RI, refractive index; LS, light scattering.

On the chromatogram obtained for sample 2 (Fig. 5) the presence of a high molecular weight fraction, corresponding to a low polymer content (i.e. a very low refractometric signal) but a large light-scattering contribution, is shown for the solution filtered through 0·2 μm. Filtration through 0·05 μm suppresses this high molecular weight peak which corresponds to 5·4% of material retained on the membrane. Then only one peak remains corresponding to $\overline{M}_w = 83\,800$. It is worth noting that the radius of gyration corresponding to the highest molecular weight signal which disappears after filtration through a 0·05 μm filter is larger than 47 nm, i.e. of the same order of magnitude or larger than the pore diameter of the filters (0·05 μm).

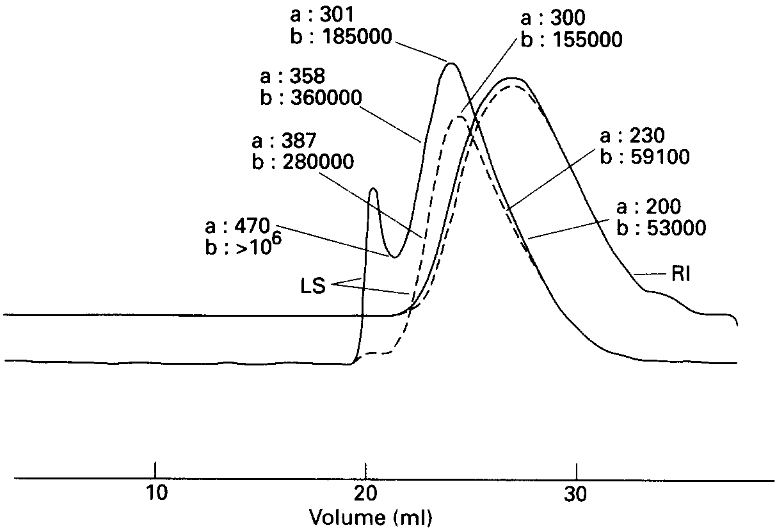

Fig. 5. Chromatograms of sample 2 after filtration through 0·2 μm (———) and 0·05 μm (- - - - -). For other symbols, see Fig. 4.

The measured radii of gyration and elution volumes at the top of the light-scattering signal (Fig. 5), are equal for the samples before and after filtration through 0·05 μm, despite the higher molecular weight of the former. This can be explained by a loss of more compact matter during the filtration process. This material is less sensitive to the shear forces applied during filtration than the isolated polymeric chains.

The light-scattering signal of sample 3 (Fig. 6) after filtration through a 0·2 μm pore size filter was decomposed into three parts and the refractometric signal into two parts (Table 3). The largest molecules are assumed to correspond to the weight loss during filtration through a 0·05 μm filter ($w_1 = 3\%$), the second fraction corresponds to the first part of the refractometric signal ($w_2 = 12\%$) and fraction 3 which corresponds to $w_3 = 85\%$ to the lower molecular weight material. After filtration through 0·05 μm, the spectra show a large decrease in the content of the largest molecules, though there is still a bimodal distribution indicating the presence of a high molecular weight fraction consisting of 15% of the material. $\overline{M}_w$ of this material is ten times larger than the lowest molecular weight species ($\overline{M}_w = 34800$). This pectate contains no calcium but has a lower content of galacturonic acid units than previous samples, and a higher neutral sugar content. According to Kravtchenko *et al.* (1992*a*, *c*) the high molecular size fraction of apple pectin could be of a different nature to that of citrus pectin. It may consist of individual molecules of a very large size containing

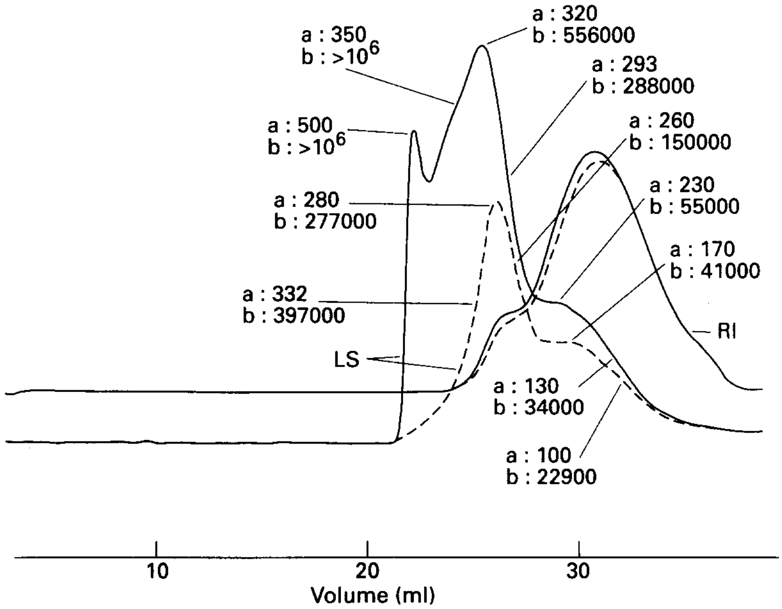

Fig. 6. Chromatograms of sample 3 after filtration through 0·2 μm (———) and 0·05 μm (- - - - -). For other symbols, see Fig. 4.

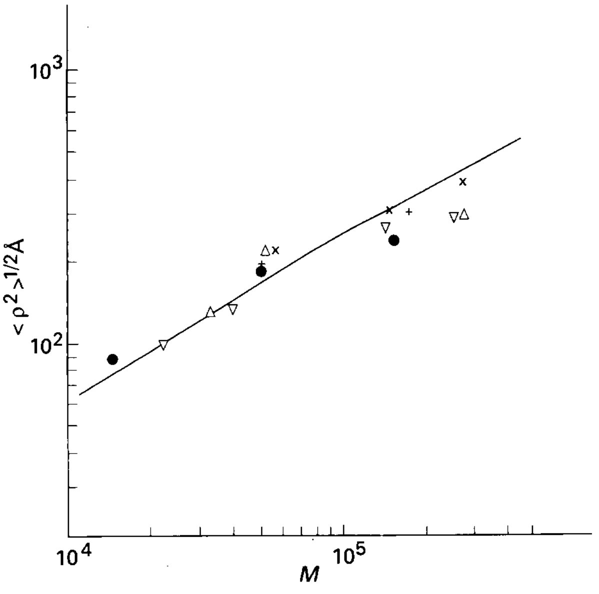

Fig. 7. $\langle\rho^2\rangle^{1/2}$ *versus* M at different elution volumes for sample 1 (●); for sample 2 after filtration through 0·2 μm (+) and 0·05 μm (×); and for sample 3 after filtration through 0·2 μm (△) and 0·05 μm (▽). The continuous line is the theoretical curve from the Benoit–Doty relation.

neutral sugars rather than aggregated molecules. $\overline{M}_w$ and the intrinsic viscosity of the largest fraction of material ($w_3 = 85\%$) is relatively low as a result of depolymerization during alkaline de-esterification. As in sample 2, the high molecular weight material with an average radius of gyration larger than 40 nm is retained in 0·05 μm filters and seems not to be shear deformed because we do not find it again after filtration.

The values of the radius of gyration and molecular weight at different positions of the peaks allow log $\langle\rho^2\rangle^{1/2}$ to be plotted against log M. This dependence is given in Fig. 7. The gradient was found to be 0·57 for the lowest molecular weight range, omitting the radius of gyration determined on large aggregates ($M > 10^5$) corresponding to more compact particles for which $\langle\rho^2\rangle^{1/2}$ increases only slowly with molecular weight. The line in Fig. 7 represents the theoretical prediction $\langle\rho^2\rangle^{1/2}$ *versus* M following the Benoit–Doty relation (Benoit & Doty, 1953) using a persistence length of 9 nm. With the exception of the highest molecular weights, especially for sample 3, there is a good agreement with experimental values. The persistence length found from this fit is in the same range as that given by Axelos and Thibault (1991) for different pectins.

In a recent paper, Berth (1992) studied the characterization of alginates and pectates by light scattering and viscometry coupled with GPC. The effect of the pore size of the membrane filters was studied for alginates where satisfactory separation of molecularly dispersed polymer from extraneous material was achieved through membranes with pore sizes of 0·2 μm. For pectates, influence of pore size of the membrane filters was not studied, but the presence of high molecular weight ($\overline{M}_w > 100\,000$) material was demonstrated. The polymer in the low molecular weight region $\overline{M}_w < 100\,000$ showed wormlike chain behaviour. This corresponds to the same behavior described in this paper showing the importance of solution prepared for obtaining reliable estimates of molecular weights, radii of gyration and persistence lengths. In contrast to the results obtained for alginates by Berth (1992), the complete removal of aggregates is not always possible on pectates even after filtration through membranes with pore sizes as low as 0·05 μm. As a consequence, the $\overline{M}_w$ of pectins are often overestimated.

REFERENCES

Anger, G. & Berth, G. (1985). *Carbohydr. Polym.*, **5**, 241–50.
Anger, G. & Berth, G. (1986). *Carbohydr. Polym.*, **6**, 193–202.
Axelos, M.A.V. & Thibault, J.F. (1991). *Int. J. Biol. Macromol.*, **13**, 77–82.
Benoit, H. & Doty, P. (1953). *J. Phys. Chem.*, **57**, 958–63.
Berth, G. (1992). *Carbohydr. Polym.*, **19**, 1–9.
Brigand, G., Denis, A., Grall, M. & Lecacheux, D. (1990). *Carbohydr. Polym.*, **12**, 61–77.
Davis, M.A.F., Gidley, M.J., Morris, E.R., Powell, D.A. & Rees, D.A. (1980). *Int. J. Biol. Macromol.*, **2**, 330–4.
Gemeiner, P., Kurillova, L., Markovic, O., Malovikova, A., Uhrin, D., Ilavsky, M., Stefuca, V., Polakovic, M. & Bales, V. (1991). *Biotechnol. Appl. Biochem.*, **13**, 335–45.
Hourdet, D. & Muller, G. (1987). *Carbohydr. Polym.*, **7**, 301–12.
Hourdet, D. & Muller, G. (1991*a*). *Carbohydr. Polym.*, **16**, 409–32.
Hourdet, D. & Muller, G. (1991*b*). *Carbohydr. Polym.*, **16**, 113–35.
Kohn, R. & Tibenský, V. (1965). *Chem. Zvesti.*, **19**, 98–106.
Kravtchenko, T.P., Berth, G., Voragen, A.G.J. & Pilnik, W. (1992a). *Carbohydr. Polym.*, **18**, 253–63.
Kravtchenko, T.P., Voragen, A.G.J. & Pilnik, W. (1992*b*). *Carbohydr. Polym.*, **18**, 17–25.
Kravtchenko, T.P., Voragen, A.G.J. & Pilnik, W. (1992*c*). *Carbohydr. Polym.*, **19**, 115–24.
Michel, F., Doublier, J.L. & Thibault, J.F. (1982). *Prog. Food Nutr. Sci.*, **6**, 367–76.
Odijk, T. & Houwaart, A.C. (1978). *J. Polym. Sci., Polym. Phys. Ed.*, **16**, 627–39.
Ravanat, G. (1979). PD Thesis, University of Grenoble, France.
Rombouts, F.M. & Thibault, J.F. (1986). *Carbohydr. Res.*, **154**, 177–87.
Tibenský, V., Rosik, J. & Zitko, V. (1963). *Nahrung*, **7**, 321–5.
Tibenský, V., & Kohn, R. (1970). Czech patent 137 935 (*Chem. Abstr.*, **77**, 105 613v, 1972).
Tinland, B., Mazet, J. & Rinaudo, M. (1988). *Makromol. Chem. Rapid Commun.*, **9**, 69–73.