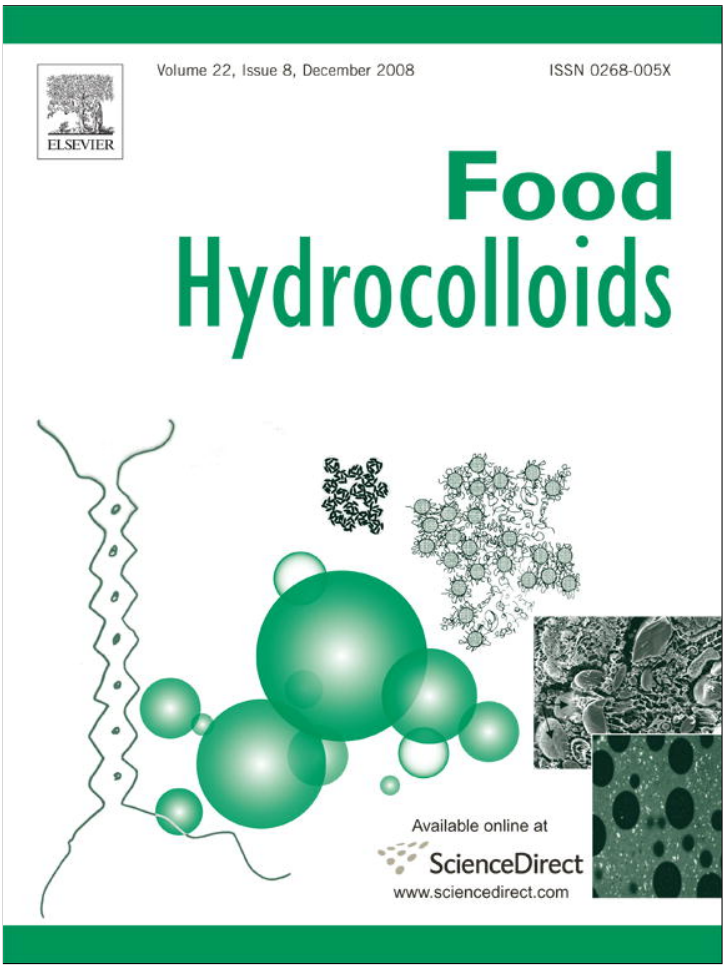

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Molecular flexibility of citrus pectins by combined sedimentation and viscosity analysis

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Abstract

The flexibility/rigidity of pectins plays an important part in their structure–function relationship and therefore on their commercial applications in the food and biomedical industries. Earlier studies based on sedimentation analysis in the ultracentrifuge have focussed on molecular weight distributions and qualitative and semi-quantitative descriptions based on power law and Wales–van Holde treatments of conformation in terms of "extended" conformations [Harding, S. E., Berth, G., Ball, A., Mitchell, J.R., & Garcìa de la Torre, J. (1991). The molecular weight distribution and conformation of citrus pectins in solution studied by hydrodynamics. *Carbohydrate Polymers*, *168*, 1–15; Morris, G. A., Foster, T. J., & Harding, S.E. (2000). The effect of degree of esterification on the hydrodynamic properties of citrus pectin. *Food Hydrocolloids*, *14*, 227–235]. In the present study, four pectins of low degree of esterification 17–27% and one of high degree of esterification (70%) were characterised in aqueous solution (0.1 M NaCl) in terms of intrinsic viscosity [η], sedimentation coefficient ($s^{\circ}_{20,w}$) and weight average molar mass (M_w). Solution conformation/flexibility was estimated qualitatively using the conformation zoning method [Pavlov, G.M., Rowe, A.J., & Harding, S.E. (1997). Conformation zoning of large molecules using the analytical ultracentrifuge. *Trends in Analytical Chemistry*, *16*, 401–405] and quantitatively (persistence length L_p) using the traditional Bohdanecky and Yamakawa–Fujii relations combined together by minimisation of a target function. Sedimentation conformation zoning showed an extended coil (Type C) conformation and persistence lengths all within the range $L_p = 10$–13 nm (for a fixed mass per unit length).

Keywords: Intrinsic viscosity; Sedimentation coefficient; Persistence length; Conformation zoning; Target function

1. Introduction

Pectins are a family of complex polyuronide-based structural polysaccharides, which constitute approximately one-third of the dry weight of higher primary plant cell walls (Tombs & Harding, 1998; Van Buren, 1991). These molecules are particularly prevalent in fruit cell walls (Ridley, O'Neil, & Mohnen, 2001; Willats, McCartney, Mackie, & Knox, 2001), especially citrus fruits and apple pommace. The main pectin chain is composed of α (1→4)-linked D-galacturonic acid residues. Many of the galacturonic acid residues have been esterified at C-6 to form methyl esters. Theoretically, the degree of esterification (DE) can range from 0% to 100% (Pilgrim, Walter, & Oakenfull, 1991). Pectins with a DE >50% are classified as high methoxyl (HM) pectins, and consequently low methoxyl (LM) pectins have a DE <50% (Pilgrim et al., 1991). Rhamnose residues are incorporated into the main chain at random intervals, which results in a kink in the otherwise linear chain (Axelos & Thibault, 1991). Side chains of arabinans and galactans are also present, either randomly dispersed or in localised "hairy" regions (Tombs & Harding, 1998). Besides the primary structure (Perez, Rodríguez-Carvajal, & Doco, 2003) (depicted in Fig. 1), the conformation and flexibility of a pectin molecule is

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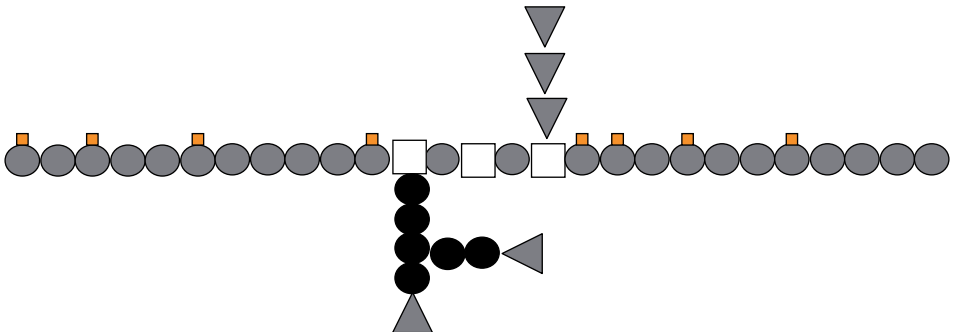

Fig. 1. Schematic structure for pectin: galacturonic acid (●); galactose (●); arabinose (▼); rhamnose (□) and methyl groups (■). Adapted from Perez et al. (2003).

important to the functional properties in the plant cell wall and also significantly affects their commercial use in the food and biomedical industries (Lapasin & Pricl, 1995; Tombs & Harding, 1998).

Previous hydrodynamic studies based on sedimentation velocity in the analytical ultracentrifuge have focussed on qualitative/semi-quantitative methods of estimating the conformation based around "power law" Mark–Houwink–Kuhn–Sakurada relations (Tombs & Harding, 1998), which link sedimentation coefficient with molar mass $s \sim M^b$ and intrinsic viscosity with molar mass $[\eta] \sim M^a$, a and b having particular values for random coils (0.5–0.8 and 0.4–0.5, respectively), spheres (0 and 0.67) and rods (1.8 and 0.15), the translational frictional ratio (Tanford, 1961) f/f_0 (minimum value of 1 for a compact unhydrated sphere) and the "Wales–van Holde" ratio (Wales & van Holde, 1954) R of the sedimentation concentration regression coefficient, k_s to the intrinsic viscosity (with limiting values of 1.6 for random coils and compact spheres and 0.15 for a rod) (Tombs & Harding, 1998). A picture of an extended conformation for pectins irrespective of DE (and charge) emerged from those studies. Morris et al. (2000) found for a series of citrus pectins of weight-average molecular weight M_w ~200,000 g/mol and DE ~30–80% in a solution of ionic strength $I = 0.1$ M, frictional ratios between 7.8 and 9.6 and R values between 0.3 and 0.8, and a suggestion of increasing flexibility with increasing DE. An earlier study (Harding, Berth, Ball, Mitchell, & Garcìa de la Torre, 1991) on fractions across a wide range of M_w (from 36,000 to 300,000 g/mol) from one particular pectin of DE ~70% in a solution of $I = 0.3$ M had given lower values for R (0.1–0.2, with one fraction ~0.7).

In this paper, we make semi-quantitative estimates of the overall solution conformation of pectins of different DE using the Wales–van Holde ratio (R) and the translational frictional ratio as before, but then use the sedimentation conformation zoning method of Pavlov and co-workers (Pavlov, Harding, & Rowe, 1999; Pavlov, Rowe, & Harding, 1997) to establish the conformation zone or type: this method requires measurement of the sedimentation coefficient, the concentration regression coefficient k_s and knowledge of the mass per unit length M_L. Finally, we make a quantitative estimate of the conformational flexibility as manifested by the persistence length L_p using a combination of the Bohdanecky (1983) and Yamakawa and Fujii (1973) representations for the intrinsic viscosities and sedimentation coefficients, respectively, of worm-like coils (Ortega & García de la Torre, 2007).

2. Materials and methods

2.1. Samples

Pectin samples (>65% galacturonic acid) and DE of 21% (P_{21}), 19% (P_{19}), 17% (P_{17}) and 27% (P_{27}) were obtained from CP Kelco (Lille Skensved, Denmark) and a further pectin (P_{70}) of DE 70% was a gift from Citrus Colloids (Hereford, UK). Pectin samples P_{17}, P_{27} and P_{70} (200 mg) were dissolved in 0.1 M NaCl (70 ml) with stirring for 16 h, whilst pectin samples P_{19} and P21 (400 mg) were dissolved in distilled water (70 ml) and exhaustively dialysed against distilled water to remove sucrose (~50% by weight) added by the manufacturer to standardise the material prior to equilibrium dialysis against 0.1 M NaCl. The resultant solutions (~3 mg/ml) were diluted to the appropriate concentrations required for biophysical characterisations.

2.2. Capillary viscometry

Solutions and reference solvents were analysed using a 2 ml automatic Schott-Geräte Oswald viscometer, under precise temperature control (25.00 ± 0.01 °C). The relative, η_{rel}, and specific viscosities, η_{sp}, were calculated as follows:

$$\eta_{rel} = \left(\frac{t}{t_0}\right)\left(\frac{\rho}{\rho_0}\right), \quad (1)$$

$$\eta_{sp} = \eta_{rel} - 1, \quad (2)$$

where t is the average (of five replicates) flow time of the pectin solution at each concentration, t_0 is the flow time for 0.1 M NaCl (88.31 s) and because of the low concentrations

used, the ratio of the density of the solution to that of solvent (ρ/ρ_0) was taken to be unity (Harding, 1997).

Measurements were made at different concentrations and extrapolated to infinite dilution using both the Huggins (1942) and Kraemer (1938) approaches:

$$\frac{\eta_{sp}}{c} = [\eta](1 + K_H[\eta]c), \tag{3}$$

$$\frac{\ln(\eta_{rel})}{c} = [\eta](1 - K_K[\eta]c), \tag{4}$$

where the intrinsic viscosity $[\eta]$ is taken as the mean of the intercepts from Eqs. (3) and (4) and K_H and K_K are the Huggins and Kraemer constants, respectively.

2.3. Sedimentation velocity in the analytical ultracentrifuge

Sedimentation velocity experiments were performed using a Beckman Instruments (Palo Alto, USA) Optima XLI Analytical Ultracentrifuge. Pectin solutions (380 μl) at six concentrations from 0.1 to 2.5 mg/ml, and 0.1 M NaCl (400 μl) were injected into the solution and reference channels of a double sector 12 mm optical path length cell. Samples were centrifuged at 45000 rpm at a temperature of 20.0 °C. Concentration profiles and the movement of the sedimenting boundary in the analytical ultracentrifuge cell were recorded using the Rayleigh interference optical system and converted to concentration (in units of fringe displacement relative to the meniscus, j) versus radial position, r (see e.g. Harding, 2005). The data were then analysed using the "least squares, ls-g(s) model" incorporated into the SEDFIT (Version 9.4b) program (Schuck, 1998, 2005). This software, based on numerical solutions to the Lamm equation, follows the changes in the concentration profiles with radial position and time and generates an apparent distribution of sedimentation coefficients in the form of $g^*(s)$ versus $s_{T,b}$, where the * indicates that the distribution of sedimentation coefficients has not been corrected for diffusion effects (see e.g. Harding, 2005).

As sedimentation coefficients are temperature and solvent dependent it is conventional to convert sedimentation coefficients (or their distributions) to the standard conditions of 20.0 °C and water using the following equation (see e.g. Ralston, 1993):

$$s_{20,w} = s_{T,b}\left[\frac{(1 - \bar{v}\rho_{20,w})\eta_{T,b}}{(1 - \bar{v}\rho_{T,b})\eta_{20,w}}\right], \tag{5}$$

where $\bar{v}$ is the partial specific volume of pectin (Morris, Foster, & Harding, 2002) (0.63 ml/g) and $\eta_{T,b}$ and $\rho_{T,b}$ are the viscosity and density of the experimental solvent (0.1 M NaCl) at the experimental temperature (20.0 °C) and $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density of water at 20.0 °C.

To account for hydrodynamic non-ideality (co-exclusion and backflow effects), the apparent sedimentation coefficients ($s_{20,w}$) were calculated at each concentration and extrapolated to infinite dilution using the following equation (Gralén, 1944; Ralston, 1993; Rowe, 1977):

$$\frac{1}{s_{20,w}} = \frac{1}{s^0_{20,w}}(1 + k_s c), \tag{6}$$

where k_s (ml/g) is the sedimentation concentration dependence or "Gralén" coefficient (Gralén, 1944).

2.4. Size exclusion chromatography coupled to multi-angle laser light scattering (SEC–MALLS)

Analytical fractionation was carried out using a series of SEC columns TSK G6000PW and TSK G4000PW protected by a similarly packed guard column (Tosoh Bioscience, Tokyo, Japan) with on-line MALLS (Harding, Vårum, Stokke, & Smidsrød, 1991; Wyatt, 1992) (Dawn DSP, Wyatt Technology, Santa Barbara, USA) and refractive index (Optilab rEX, Wyatt Technology, Santa Barbara, USA) detectors. The eluent (0.1 M NaCl) was pumped at 0.8 ml/min (PU-1580, Jasco Corporation, Great Dunmow, UK) and the injected volume was 100 μl (~2.0 mg/ml) for each sample (in triplicate). Absolute weight-average molar masses (M_w) were calculated using the ASTRA® (Version 5.1.9.1) software (Wyatt Technology, Santa Barbara, USA), using the refractive index increment, $dn/dc = 0.146$ ml/g for pectin (Chapman, Morris, Selvendran, & O'Neill, 1987).

3. Results and discussion

3.1. Capillary viscometry

Intrinsic viscosities (Table 1) in the range 325–600 ml/g are in general agreement with previous studies on citrus pectins (Cros, Garnier, Axelos, Imbery, & Perez, 1996; Fishman, Chau, Kolpak, & Brady, 2001; Harding, Berth et al., 1991; Harding, Vårum et al., 1991; Morris et al., 2000, 2002; Ralet, Bonnin, & Thibault, 2001; Yoo, Fishman, Hotchkiss, & Lee, 2006). It can also be seen that the intrinsic viscosity decreases with decreasing DE.

3.2. Sedimentation velocity in the analytical ultracentrifuge

The ls-g(s*) plots for each pectin sample show that the samples are homogeneous (Fig. 2). The distribution of sedimentation coefficients (not corrected for buffer effects) shows a maximum at ~2.0 S and after correction for buffer

Table 1
Solution properties for pectin in 0.1 M NaCl

Pectin	DE (%)	M_w (g/mol)	$s^o_{20,w}$ (S)	$[\eta]$ (ml/g)
P_{17}	17	145,000 ± 5000	2.02 ± 0.07	325 ± 10
P_{19}	19	165,000 ± 5000	2.13 ± 0.05	395 ± 20
P_{21}	21	175,000 ± 5000	2.08 ± 0.08	400 ± 10
P_{27}	27	195,000 ± 5000	2.04 ± 0.04	495 ± 15
P_{70}	70	180,000 ± 5000	2.08 ± 0.07	600 ± 30

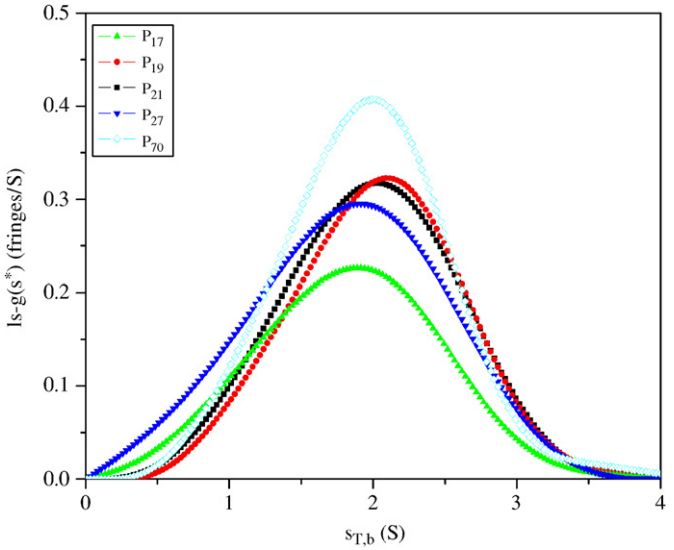

Fig. 2. ls-g(s*) distribution for pectins; P_{17} ▲ ($c = 0.15$ mg/ml); P_{19} ● ($c = 0.19$ mg/ml); P_{21} ■ ($c = 0.20$ mg/ml); P_{27} ▼ ($c = 0.21$ mg/ml) and P_{70} ◇ ($c = 0.23$ mg/ml).

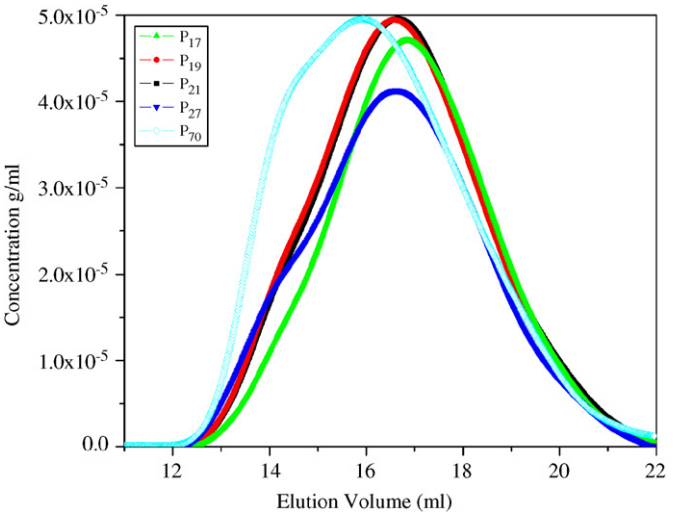

Fig. 3. Concentration profiles for pectins; P_{17} ▲ ($c = 1.86$ mg/ml); P_{19} ● ($c = 2.06$ mg/ml); P_{21} ■ ($c = 2.06$ mg/ml); P_{27} ▼ ($c = 1.77$ mg/ml) and P_{70} ◇ ($c = 2.32$ mg/ml).

effects and extrapolation to infinite dilution all pectins have a sedimentation coefficient of ~2.1 S (Table 1), which is in good agreement with previous studies (Harding, Berth et al., 1991; Harding, Vårum et al., 1991; Morris, Foster, & Harding, 1999; Morris et al., 2000, 2002).

3.3. Size exclusion chromatography coupled to multi-angle laser light scattering (SEC–MALLS)

Molar mass estimates are in broad agreement with previous estimates for citrus pectins (Corredig, Kerr, & Wicker, 2000; Cros et al., 1996; Fishman et al., 2001; Morris et al., 2000, 2002; Yoo et al., 2006). All materials appeared to be eluted in the void/total volume limits. A single, wide peak at the elution volume ~12–22 ml is evident for all samples (Fig. 3). There appears to be some difference in molar masses (Table 1) with pectin P_{17} being of slightly lower molar mass, which is consistent with a lower intrinsic viscosity, and although pectins P_{21}, P_{19}, P_{27} and P_{70} have similar molar masses pectin P_{70} elutes slightly earlier in agreement with a higher intrinsic viscosity.

4. Conformational analysis

4.1. The translational frictional ratio, f/f_0

The translational frictional ratio (Tanford, 1961), f/f_0, is a parameter that depends on conformation and molecular expansion through hydration effects. It can be measured experimentally from the sedimentation coefficient and molecular weight:

$$\frac{f}{f_0} = \frac{M_w(1-\bar{v}\rho_{20,w})}{(N_A 6\pi\eta_{20,w}s^0_{20,w})}\left(\frac{4\pi N_A}{3\bar{v}M_w}\right)^{1/3}. \quad (8)$$

Large translational frictional ratios are found for all the pectins studied, and all in the region of 7–9 (Table 2).

Table 2
Conformational parameters for pectin in 0.1 M NaCl

Pectin	f/f_0	$k_s/(\eta)$	L_p (nm)
P_{17}	7.1±0.4	0.60±0.10	10±2
P_{19}	7.3±0.3	0.65±0.10	10±2
P_{21}	7.8±0.4	0.55±0.10	11±2
P_{27}	8.6±0.4	0.40±0.05	13±3
P_{70}	8.0±0.5	0.45±0.10	13±3
Overall	7.8±0.5	0.53±0.10	12±1
Morris, Foster, & Harding (2000)	8.8±0.5	0.56±0.08	15±1

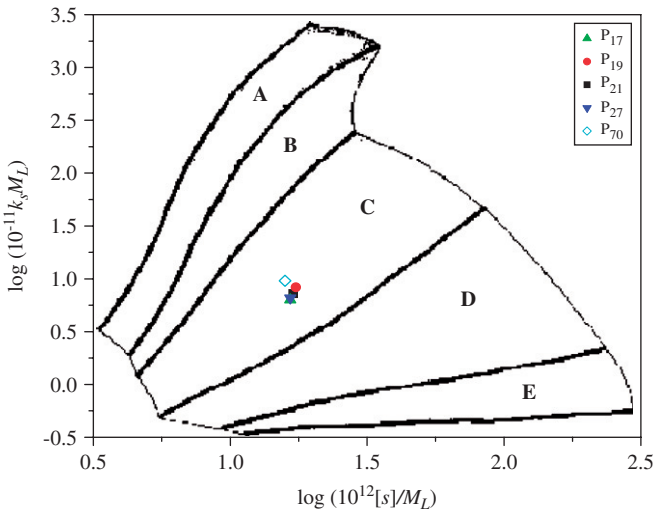

Fig. 4. Sedimentation conformation zoning plot (adapted from Pavlov et al., 1977). Zone A: extra rigid rod; Zone B: rigid rod; Zone C: semi-flexible; Zone D: random coil and Zone E: globular or branched. Individual pectins are marked: P_{17} ▲; P_{19} ●; P_{21} ■; P_{27} ▼ and P_{70} ◇.

These values are in agreement with previous estimates for citrus pectin (Morris et al., 2000, 2002) and symptomatic of expanded extended conformations for these molecules.

4.2. Wales–van Holde ratio, R

Values in the range 0.45–0.65 are obtained for $R = k_s/[\eta]$, again consistent with extended structures (Morris et al.,

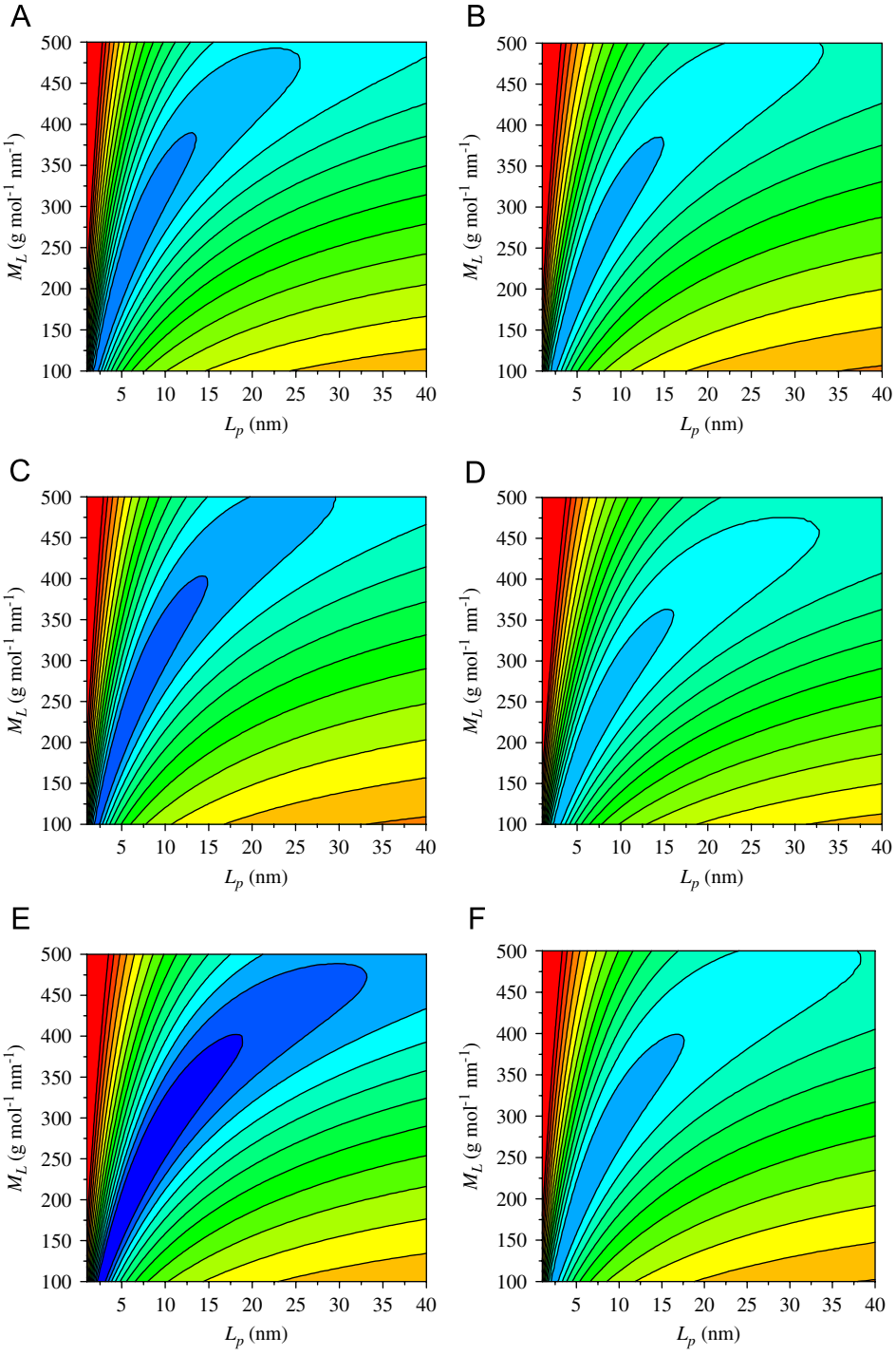

Fig. 5. *HYDFIT* analysis for pectin. Solution of the Bohdanecky (1983) and Yamakawa–Fujii (1973) relations for L_p with M_L allowed to float. The *x*-axis and *y*-axis represent L_p (nm) and M_L (g/mol/nm), respectively. The target function, $\triangle$ is calculated over a range of values for M_L and L_p. In these representations, the values of $\triangle$ function are represented by the full colour spectrum, from blue ($\triangle = 0.15$) to red ($\triangle \geqslant 1$). (A) Contour plot from *HYDFIT* analysis for pectin P_{17}. (B) Contour plot from *HYDFIT* analysis for pectin P_{19}. (C) Contour plot from *HYDFIT* analysis for pectin P_{21}. (D) Contour plot from *HYDFIT* analysis for pectin P_{27}. (E) Contour plot from *HYDFIT* analysis for pectin P_{70}. (F) Overall contour plot from *HYDFIT* analysis for pectins.

2000) but short of the limit for rod found for one pectin (0.15) (Harding, Berth et al., 1991; Harding, Vårum et al., 1991).

4.3. Sedimentation conformation zoning

The sedimentation conformation zone (Pavlov et al., 1997, 1999) plot $k_s M_L$ versus $[s]/M_L$ enables an estimate of the "overall" solution conformation of a macromolecule in solution ranging from Zone A (extra rigid rod) to Zone E (globular or branched). The parameter $[s]$ is related to the sedimentation coefficient by the relation

$$[s] = \frac{s^0_{20,w}\eta_{20,w}}{(1-\bar{v}\rho_{20,w})}, \tag{9}$$

and M_L the mass per unit length is just

$$M_L = \frac{m}{l}. \tag{10}$$

The average mass of pectin monomer, m, is 176 g/mol for anhydrogalacturonic acid (Norziah, Fang, & Abd Karim, 2000) and will be ~180 and ~190 g/mol for LM (DE 20%) and HM (DE 75%) pectins, respectively, and l is the diameter of a monosaccharide (Picout, Ross-Murphy, Errington, & Harding, 2001) ~0.54 nm, assuming no significant branching. As can be seen from the sedimentation conformation zoning plot (Fig. 4) all five pectins have an extended or "semi-flexible" conformation (Zone C). However, to obtain more quantitative information about the flexibility of the pectins, we need to consider the way in which the sedimentation coefficient and intrinsic viscosity change with molecular weight.

4.4. Combined analysis method (HYDFIT)

The linear flexibility of polymer chains is represented in terms of the persistence length, L_p, of equivalent *worm-like chains* (Kratky & Porod, 1949), where the persistence length is defined as the average projection length along the initial direction of the polymer chain and for a theoretical perfect random coil (Tombs & Harding, 1998) $L_p = 0$ and for the equivalent extra-rigid rod (Harding, 1997) $L_p = \infty$, although in practice limits of ~1 nm for random coils (e.g. pullulan) and 200 nm for an extra-rigid rod (e.g. DNA) are more appropriate (Tombs & Harding, 1998). Chain persistence lengths, L_p, can be estimated using several different approaches using either intrinsic viscosity (Bohdanecky, 1983; Hearst, 1963; Stockmayer & Fixman, 1963) or sedimentation coefficient (Hearst & Stockmayer, 1962; Yamakawa & Fujii, 1973) measurements. For example, the Bohdanecky (1983) relation

$$\left(\frac{M_w^2}{[\eta]}\right)^{1/3} = A_0 M_L \Phi^{-1/3} + B_0 \Phi^{-1/3}\left(\frac{2L_p}{M_L}\right)^{-1/2} M_w^{1/2}, \tag{11}$$

where Φ is the Flory–Fox constant (2.86×10^{23} mol^{-1}) and A_0 and B_0 are tabulated coefficients (Bohdanecky, 1983, and the Yamakawa and Fujii (1973) equation:

$$s^0 = \frac{M_L(1-\bar{v}\rho_0)}{3\pi\eta_0 N_A}\times\left[1.843\left(\frac{M_w}{2M_L L_p}\right)^{1/2} + A_2 + A_3\left(\frac{M_w}{2M_L L_p}\right)^{-1/2} + \cdots\right]. \tag{12}$$

Yamakawa and Fujii (1973) showed that A_2 can be considered as, $\ln(d/2L_p)$ and $A_3 = 0.1382$ if L_p is much higher than the chain diameter, d. Difficulties arise if the mass per unit length is not known, although both relations have now been built into an algorithm Multi_HYDFIT (Ortega & García de la Torre, 2007), which estimates the best values—or best range of values of L_p and M_L based on minimisation of a target function Δ. An estimate for the chain diameter d is also required, but extensive simulations have shown that the results returned for L_p are relatively insensitive to the value chosen for d (taken here as 0.8 nm for each pectin). The blue contour in Fig. 5A–E represents the minimum in the target function within experimental error and a continuum of possible values for L_p up to 15 nm and mass per unit lengths M_L up to 400 g/mol/nm are allowed within experimental error. However if we use the same values of M_L used for the conformation zoning plots, a more specific value for L_p can be found for each pectin. Using M_L fixed at 330 and 350 g/mol/nm for LM pectins and HM pectin, respectively, the results are shown in Table 2 and Fig. 6: a persistence length of 10–13 nm for all the pectins are returned, which agree well with previous estimates from computer modelling (Braccini, Grasso, & Perez, 1999; Cros et al., 1996; Noto, Martorana, Bulone, & San Biagio, 2005).

The Multi_HYDFIT (Ortega & García de la Torre, 2007) approach also allows for the estimation of the average persistence length for a homologous series of

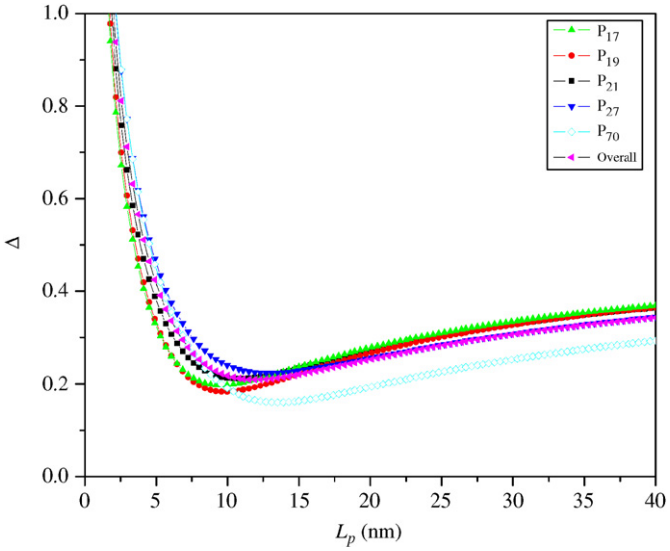

Fig. 6. *HYDFIT* analysis for pectic polysaccharides. Solution of the Bohdanecky (1983) and Yamakawa–Fujii (1973) relations for the persistence length L_p (for a known mass per unit length M_L of (330–350) g/mol/nm). Plot of target function (△) vs. persistence length for pectins: P_{17} ▲; P_{19} ●; P_{21} ■; P_{27} ▼; P_{70} ◊ and overall ◀.

polymers using sets of $s^0_{20,w}$ and $[\eta]$ versus M_w (Figs. 5F and 6). Although in this case we are not strictly speaking considering a homologous series it is clear that as all pectin molecules are of similar conformation we can justify this approach and we have furthermore extended this to the pectins studied by Morris et al. (2000), which also have a Zone C-type conformation (plots not shown). Again, results agree well with one another and with previous estimates from computer modelling (Braccini et al., 1999; Cros et al., 1996; Noto et al., 2005).

An extended coil-type (Zone C) conformation for citrus pectins of high galacturonan (>65%) content seems likely, but it should be noted that pectin conformation will depend not only on degree of methyl esterification but also on the distribution of methyl ester groups (i.e. block-wise or random) galacturonan content and on the degree of branching by neutral sugars (e.g. galactose and arabinose). Therefore, we may expect to see quite different solution conformations for more heavily branched pectins (e.g. from sugar beet and apple).

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