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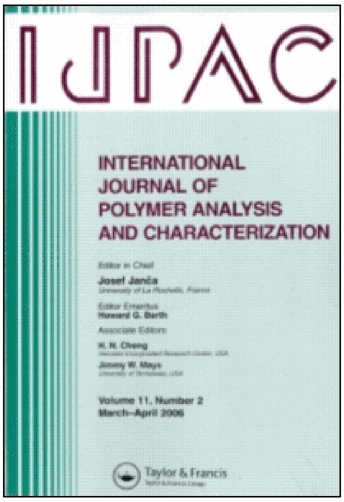

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On-line Size Exclusion Chromatography and Multiangle Laser Light Scattering of High-Molecular-Weight Rigid Polysaccharides

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High-molecular-weight "rigid" polysaccharides from bacterial origin, namely xanthan and schizophyllan, were studied in 0.1M $LiNO_3$ solutions by applying a size exclusion chromatographic system in combination with a multiangle laser light scattering detector and a refractive index detector. Both the light scattering and the elution behavior show peculiarities indicating the presence of particles of large size (microgels and/or aggregates). This complicates the interpretation of experimental data and explains why the radius of gyration scales with the molecular weight with a slope much smaller than that expected for molecularly dispersed molecules. Partial removing of such contaminants as a result of sonifying and/or ultrafiltration treatments yields polysaccharide solutions of improved quality.

KEY WORDS Size exclusion chromatography, laser light scattering, polysaccharides

INTRODUCTION

Functional properties of polysaccharides in many applications are largely due to their ability to control the rheology of aqueous media, thus they have wide applications as thickening and gelling agents in the food and oil industries. Many factors govern the functional properties of polysaccharides: the molecular size (radius of gyration Rg) and the chain conformation (characterized by the dependence of Rg on M_w). A reliable method of determination of these factors is of primary importance for understanding the relationship between the macroscopic properties and the molecular structure of biopolymers.

Although difficulties exist for water-soluble biopolymers, as compared to non-water soluble synthetic polymers [1], the application of size exclusion chromatography (SEC) coupled to on-line low angle (LALLS) or multiangle (MALLS) laser light scattering probably represents the most useful method for polysaccharide characterization [2–9].

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Nevertheless, in characterizing water-soluble polymers, and this is particularly true for high-molecular-weight polysaccharides, we are confronted with problems which markedly complicate both size exclusion and light scattering behavior. Such problems have many origins mainly related to the nature of polysaccharide molecules which present large deviations from ideal behavior due to high exclusion volumes [10]. Moreover, polysaccharides are heterogeneous systems which generally contain non-molecular species (aggregates, gel particles and/or microgels) and/or low-molecular-weight impurities (proteins), thereby considerably complicating the analysis of experimental data.

The aim of this paper is to present and discuss some problems associated with the molecular characterization of two high-molecular-weight rigid polysaccharides, xanthan and schizophyllan, by on-line SEC and MALLS. These polymers show very interesting and unusual rheological properties related to the inherent stiffness of their backbone. This explains why xanthan has been the object of considerable interest in recent years and has been used in a wide range of applications [11]. Moreover both polysaccharides can be considered as good examples of the role played by "minor components" in the overall properties of polysaccharide chains [12].

EXPERIMENTAL SECTION

Materials

Xanthan is an exocellular, anionic heteropolysaccharide secreted by *Xanthomonas campestris* and consists of a β-(1–4)-D-glucose backbone with trisaccharide side-chains (1–3)-linked to alternate backbone residues with a pyruvate substituent carried by the terminal mannose. A powdered xanthan sample recovered from a culture broth (SBI, France) by precipitation in isopropyl alcohol was investigated (extent of pyruvate substitution was 0.5–0.6).

The schizophyllan sample was kindly supplied by Taito Company Ltd (Japan). The sample was slightly degraded by hydrodynamic shear treatment. Schizophyllan is a neutral polysaccharide produced by the fungus *Schizophyllum commune* and consists of linearly linked β-(1–6)-D-glucopyranoside side-chain for every three main-chain residues.

The following procedure was used for obtaining solutions of purified schizophyllan free of low-molecular-weight species. The solutions (c = 3g/L) prepared by direct dissolution of the powder were successively filtrated from 8 to 0.8μm, then submitted to extensive ultrafiltration at constant volume for 20 days against distilled water containing NaN_3 as a bactericide (Amicon Membrane, M_w cutt-off 100,000g/mol).

Laser Light Scattering and Size Exclusion Chromatography

A Chromatix KMX-6 (LDC Analytical) light scattering apparatus was used for determining the molecular weight M_w and the second virial coefficient A_2 from measurements of the excess Rayleigh factor R_θ at an angle sufficiently small ($\theta = 5°$) so that the angular dependence is totally eliminated ($P(\theta) = 1$ in Equation 1).

A multiangle laser light scattering photometer equipped with a flow cell (Dawn F, Wyatt Technology) in series with a concentration sensitive detector (Shimadzu RID-6A) was used as a light scattering detector for size exclusion chromatography. Analytical

SEC/MALLS experiments were performed using two serially connected columns (TSK 6000PW and TSK 4000PW) and eluted (Kontron HPLC pump 6420, 0.7mL/min) with filtered (GS 0.22 μm) 0.1M $LiNO_3$. The injected volume was 400 μL and the polymer concentration 0.2 g/L.

The refractive index increment dn/dc was measured in 0.1M NaCl with a Brice Phoenix differential refractometer and was 0.143 mL/g for xanthan and schizophyllan.

Data collection from the Dawn F and the DRI detector was controlled using Wyatt Technology ASTRA program (V.3.0) and the results were analysed using EASI (V.7.0) software package.

The general expression for the excess Rayleigh factor is:

$$R_\theta = \mathrm{KcMP}(\theta)\,[1 - 2A_2\mathrm{MP}(\theta)c] \qquad (1)$$

where M_w and the radius of gyration Rg are extracted from the intercept and the slope near the origin of R_θ/Kc (Debye plot) or Kc/R_θ (Zimm plot) versus $\sin^2\theta/2$, and

$$P(\theta) = 1 - 1/3\, q^2 Rg^2 \qquad (2)$$

where q is the scattering vector equal to $(4\pi n/\lambda)\sin\theta/2$.

The molecular characteristics of both biopolymers are given in Table I.

RESULTS AND DISCUSSION

Off-line static light scattering has proven to be particularly useful for polymer size determination, but has inherent difficulties when applied to high M_w polysaccharides. Owing to these difficulties, light scattering experimental data should be interpreted with caution to avoid erroneous interpretation. Firstly, the need for molecularly dispersed, high-quality solutions free of dust and traces of large aggregates makes necessary the use of clarification procedures, such as centrifugation, filtration, ultrafiltration, etc., which can affect solution properties. Secondly, an exact extrapolation to zero angle and zero concentration is necessary to determine M_w and Rg. This is a difficult experimental problem for large molecules or for polydisperse systems of large molecules because if the sample contains a small amount of aggregates or gel particles, the angular dependence of the scattered light exhibits marked curvature in the small angle range. The main limitation of off-line light scattering systems is the lack of information on the MWD; this is now overcome by combination with SEC.

TABLE I

Molecular characteristics of polysaccharide samples in 0.1 M NaCl

Samples	M_w (g/mol)	A_2 (mL · mol/g2)	η (mL/g)***
Xanthan	5.3×10^6* 4.9×10^6**	5.1×10^{-4}	7150
Schizophyllan	2.0×10^6*	$\cong 0$	3000

*LALLS using the Chromatix model KMX-6
**MALLS using a Dawn F
***Low-shear viscosity using Contraves LS-30.

However, on-line SEC/LALLS and MALLS analysis of high M_w polysaccharides introduces additional difficulties and limitations regarding the efficiency of available packing materials for the chromatographic columns in the presence of eluted molecules with large hydrodynamic volumes. This difficulty influences the reliability of the analytical treatment of the data. These concerns are illustrated and discussed in this paper.

Figures 1 and 2 show typical three-dimensional plots of the scattered light intensity from high M_w native xanthan and schizophyllan molecules, respectively, over an angular range of about 15° (detector 3) to 160° (detector 18). From these chromatograms both M_w and Rg at each retention volume are provided upon construction of a Zimm and/or a Debye plot. A typical Debye plot is given in Figure 3. As reported by others [13,14], for large dissymetrical molecules with $P(\theta)<<1$, many problems arise concerning the determination of M_w and *Rg* from the angular dependence of the scattered light. Generally, we observed that the Debye method (with a high-order polynomial fit) yields more realistic M_w and *Rg* values than the Zimm method, provided that one takes into account experimental error and the type of polymer in solution (i.e., conformation, presence of contaminants/microgels, etc.) for a judicious selection of the angular expansion. Two facts are worth mentioning from examination of chromatograms of Figures 1 and 2. Firstly, the noise level increases with decreasing scattering angles. Secondly, the scattering behavior in the small-angle range and, more precisely, the strong curvature of the scattering envelope reveals the presence of "contaminants" which strongly disturb the entire scattering behavior. This can lead to erroneous values of both M_w and *Rg* and seriously complicate

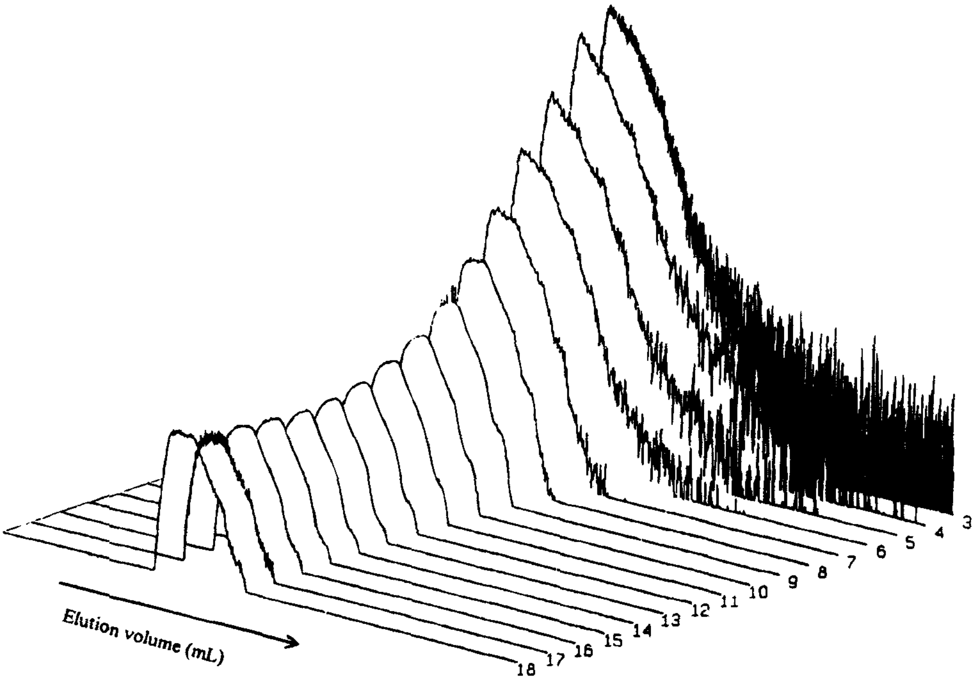

FIGURE 1 Three-dimensional plot of normalized scattered light intensity from xanthan at different scattering angles between 15° (detector 3) and 160° (detector 18). (RI refers to the refractive index signal).

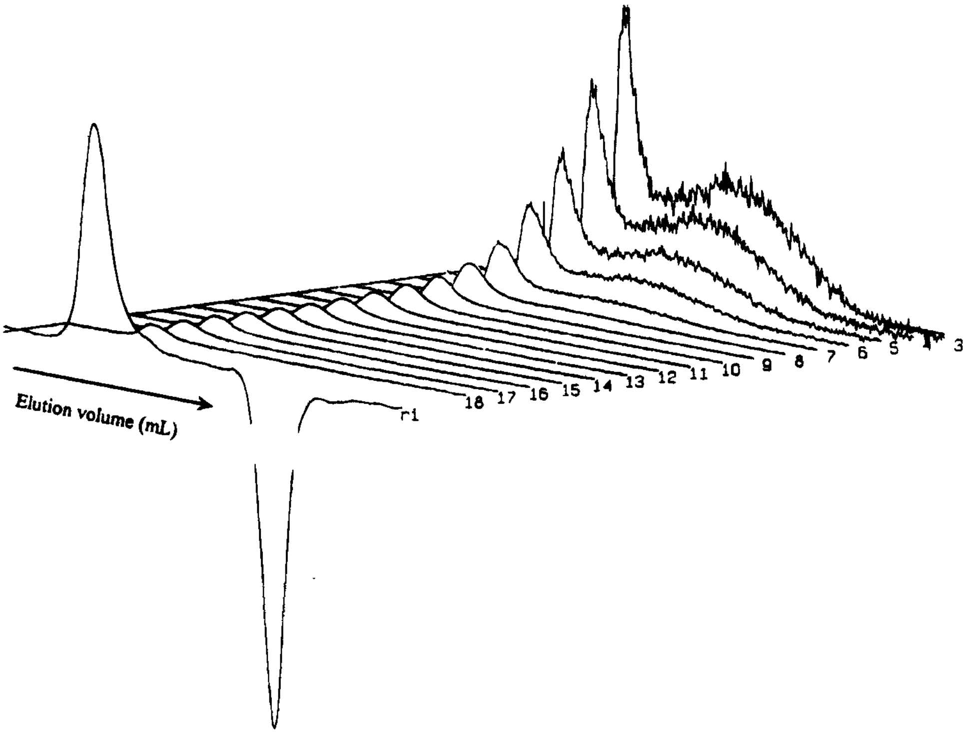

FIGURE 2 Three-dimensional plot of normalized scattered light intensity for a native schizophyllan at different scattering angles between 15° (detector 3) and 160° (detector 18). (RI refers to the refractive index signal).

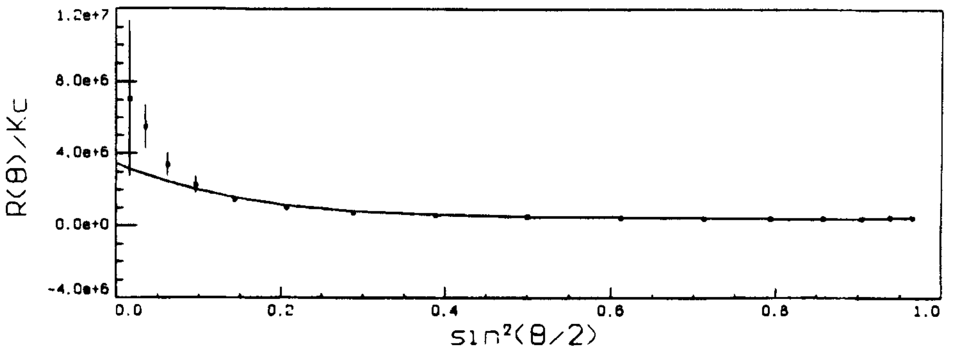

FIGURE 3 Angular dependence of the scattering intensity measured at the top of the elution peak of native xanthan sample (debye plot with a fourth-order fit).

Because of the curvature in the small-angle range, the extrapolation to zero angle is ambigous.

As calculated by the Debye plots reported in Figure 4, both the calculated M_w and Rg are found dependent on the order of the fitting polynomial. Therefore, selection of the appropriate order necessary to fit experimental data for such high M_w and size molecules

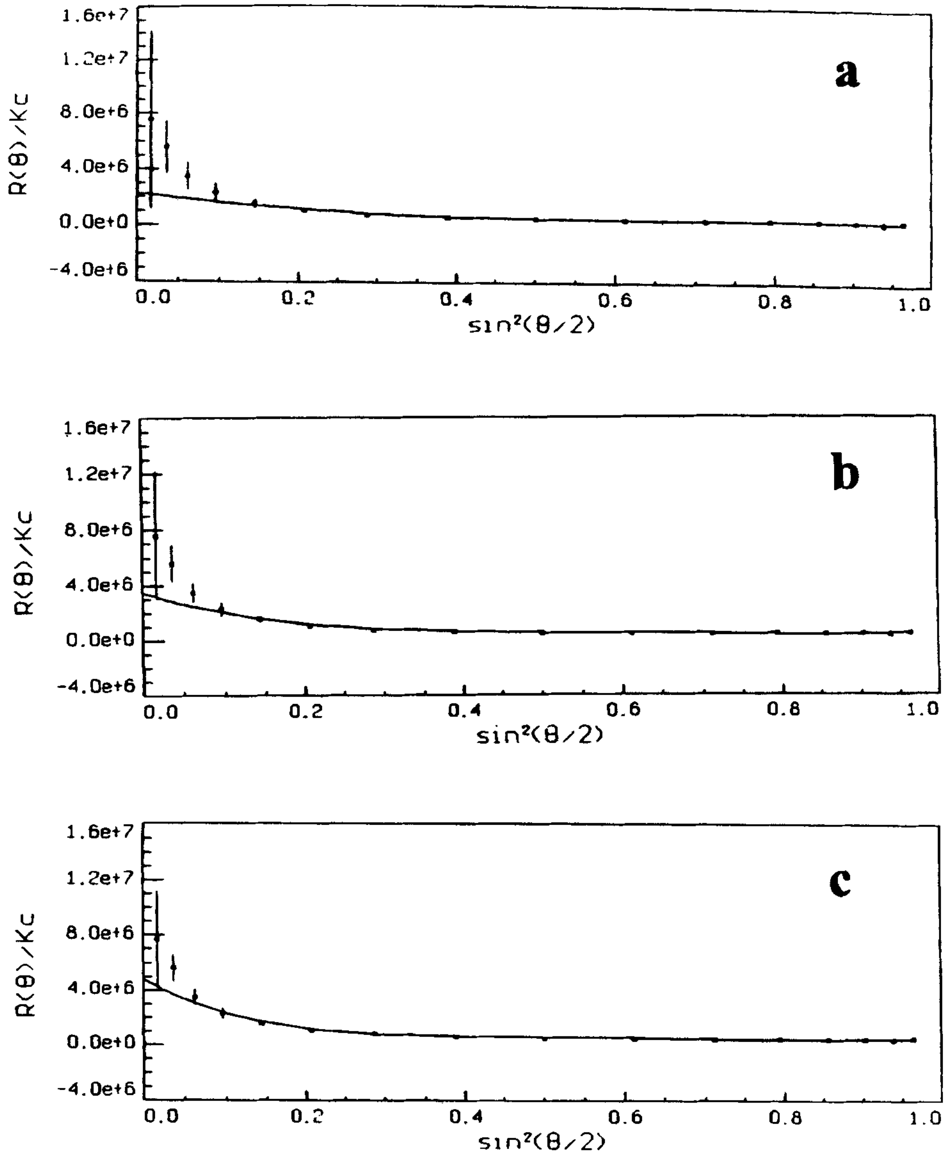

FIGURE 4 Debye plots for native xanthan solution: effect of the order of the fit on the calculated molecular weight (a, b, c, correspond to order 3, 4, and 5, respectively).

requires attention and a good appreciation of the relevance of the lowest angle data scattered intensities. For xanthan, we found that a fourth-order fit was the most appropriate. The SEC/MALLS data were compared to experimental data obtained from LALLS measurements using the Chromatix KMX-6. It is well known that the two extrapolated curves of scattered light, as a function of both concentration and angle, should have a common intercept yielding $1/M_w$.

At low forward angle ($\theta = 5°$, Chromatix), the excess Rayleigh factor varies linearly with concentration with a slope of $2A_2$. This slope is generally slightly concentration dependent. However, in SEC/MALLS, M_W is calculated from the angular dependence of the scattered light, a quantity which shows a noticeable curvature and therefore varies with angle with a slope much steeper than $2A_2$. Independent of the noise, which increases with decreasing angle, this curvature makes it difficult to extrapolate to zero angle, especially for high M_W polymers containing microgels or other contaminants, as slight errors in the fit well cause large errors in the calculated M_W and Rg values. In the case of native xanthan, illustrated by data in Figure 4, it is quite clear that overestimation (plot c) or underestimation (plot a) of the molecular weight results whether the lowest angle values are taken into consideration or not.

Our experience indicates that the LALLS data could be a great help for the analysis of MALLS data particularly if some contaminants are present in polymer solutions. Such contaminants together with the large size of the polymer molecules are responsible for the unusual effects observed in the elution behavior of both native biopolymers. Figure 5 shows the behavior of schizophyllan. The molecular weight and the radius of gyration of both biopolymers as a function of elution volume show that the initial and usual decrease of M_w and Rg at small elution volume (Ve) is followed by an increase

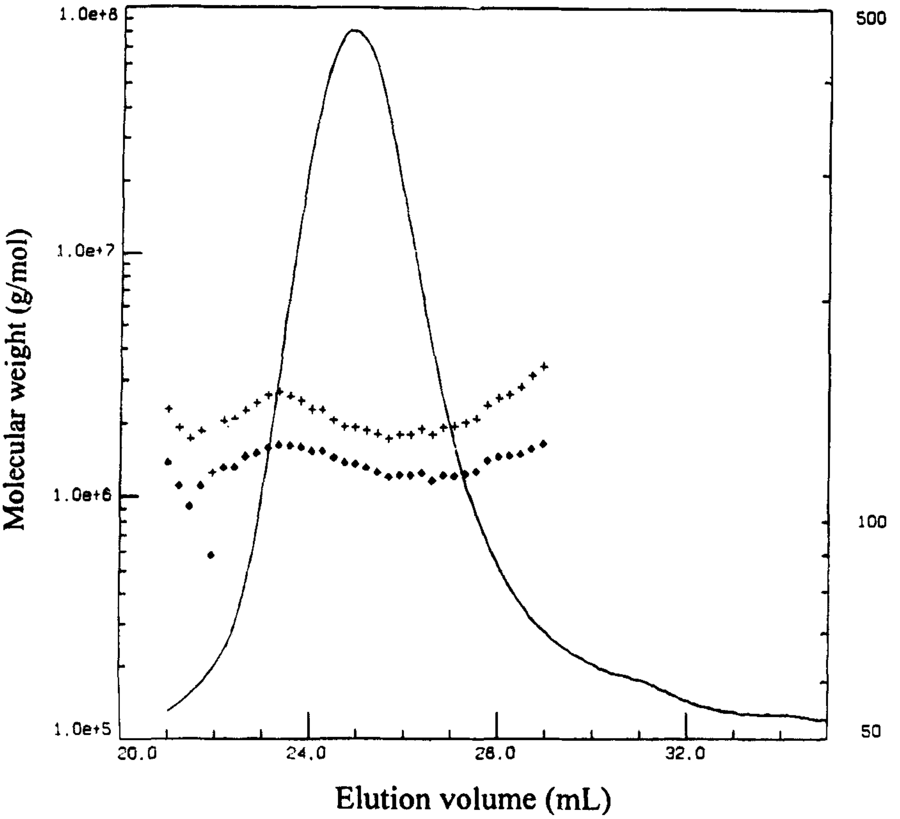

FIGURE 5 Radius of gyration (nm) (+ +) and molecular weight (◆ ◆) as a function of elution volume for a native schizophyllan.

of these parameters for larger volumes. Similar SEC/MALLS behavior has been reported for microgel-contaminated polymers clearly showing that the presence of supramolecular structures is responsible for the unexpected elution behavior [16–18]. Obviously, unexpected "size" separation effects greatly complicate the elution behavior of such non-molecular dispersed polysaccharides. Even by taking into account the difficulty of knowing precisely the exact concentration of the small RI signal at both ends of the distribution (and therefore the exact M_w and Rg values), such a general elution behavior could be ascribed to the polydispersity of poorly defined desorbing species ("microgels").

Many attempts have been made to obtain well-defined biopolymer solutions. Reducing molecular weight of xanthan by ultrasonic degradation limits the above reported effects responsible for the wrong dependence of *Rg* and M_w on the elution volume (Figs. 6 and 7). However, the light scattering data reveal a more symmetrical envelope particularly in the small-angle range (Fig. 8). This is a consequence of two factors: reducing the size of the molecule and partial destruction of microgels. As shown in Figure 6, the radius of

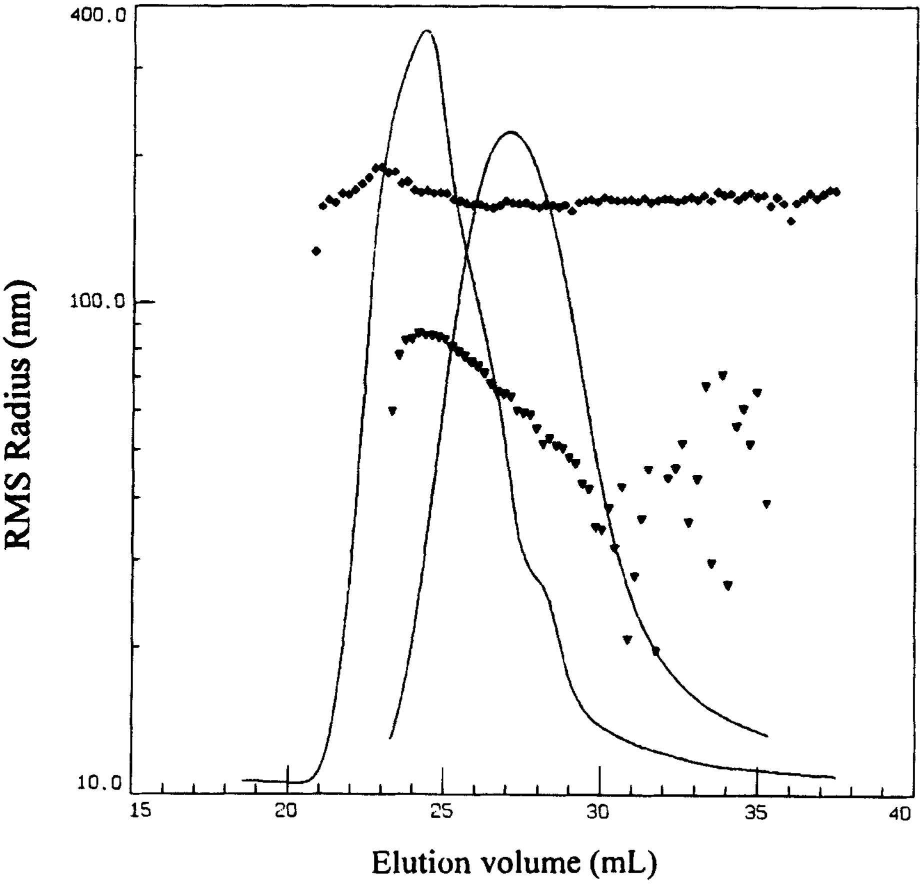

FIGURE 6 Dependence of the radius of gyration (nm) on the elution volume for a xanthan sample: native (◆ ◆) and after sonication for 15 min (▼ ▼).

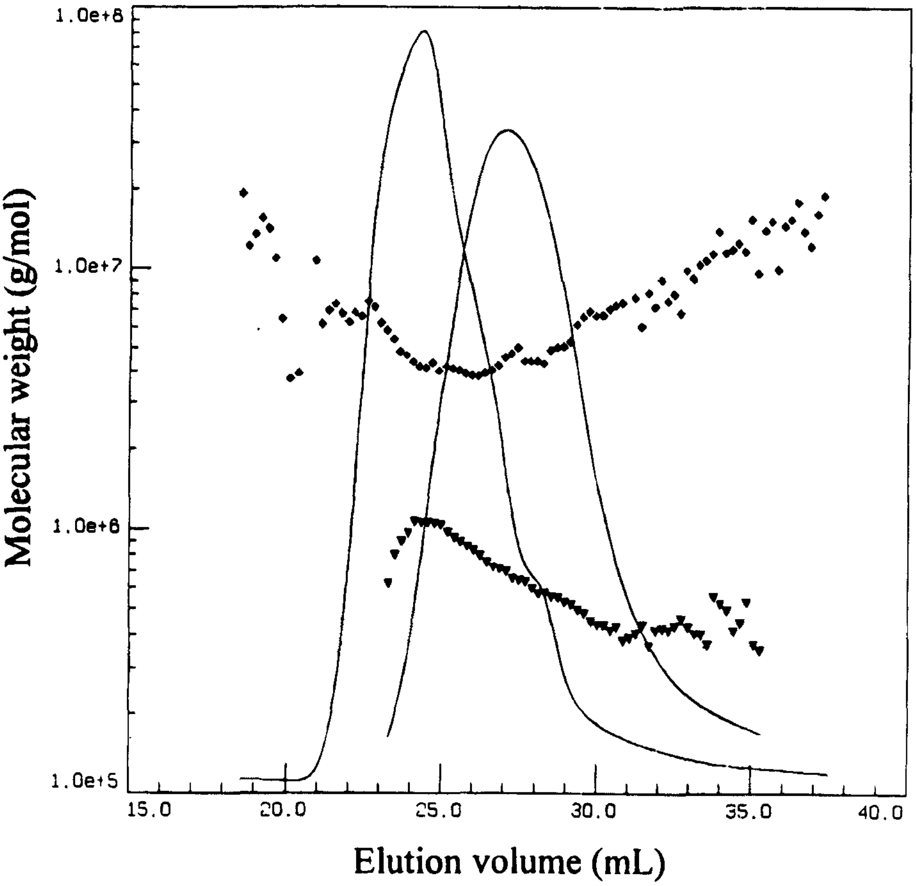

FIGURE 7 Effect of ultrasonic treatment on the molecular weight versus elution volume behavior of xanthan: (◆ ◆) native (▼ ▼) after sonicaton 15 min.

gyration of sonicated sample as a function of eluted volume shows an expected behavior. Moreover the dependence of the radius of gyration on the molecular weight reflects that microgels (even if present in a small quantity) do not disturb the eluted ultrasonically degraded fractions since the slope of $Rg{\sim}M_w^x$ is much higher (0.9) than that of the native sample (0.37, a value expected for a compact conformation) (Table II).

In the case of schizophyllan, extensive ultrafiltration treatment against distilled water resulted in well defined molecularly dispersed polysaccharide solution. Figure 9 shows the effect of ultrafiltration on the molecular weight distribution of schizophyllan. A narrower distribution is observed for the treated sample. It is interesting to note from the analysis of the elution behavior of schizophyllan that part of the compact supramolecular structures (low Rg but large M_w) responsible for the above reported SEC behavior at high elution volume has been removed as a result of ultrafiltration. However low-molecular-weight contaminants (e.g., proteins), which can act as "cross-linkers", are probably eliminated during the treatment thus accounting for reduced aggregation. Ultrafiltration simplifies the treatment of light scattering data and indicates that the presence of supramole-

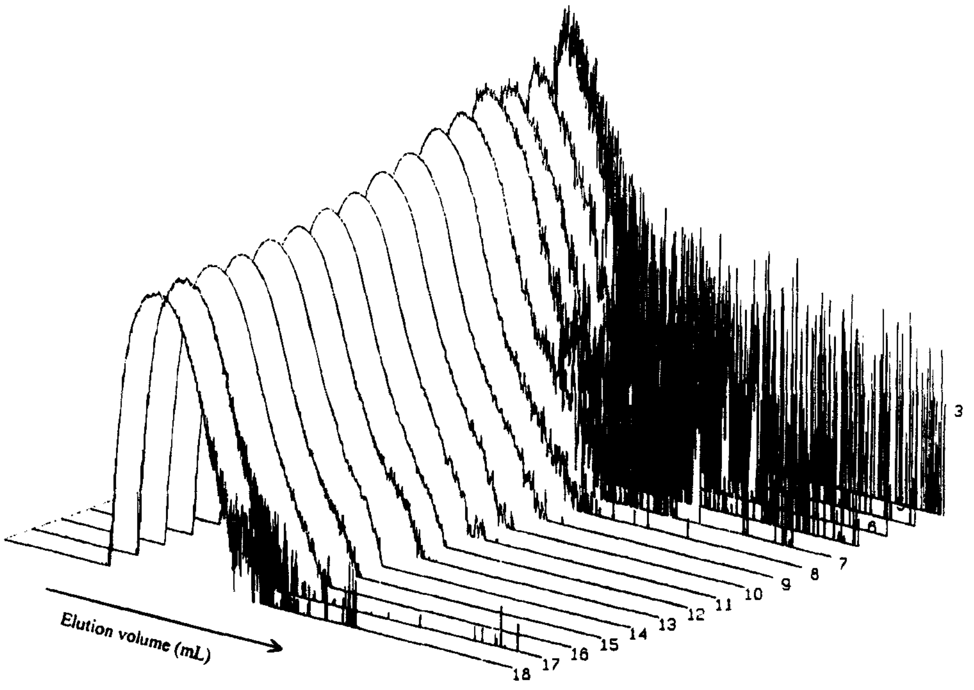

FIGURE 8 Scattering behavior of a sonicated xanthan sample.

TABLE II

Comparison of native and degraded xanthan

Sonication (min)	M_w (g/mol)	Rg (nm)	x
0	1.32×10^6	106	0.37
5	1.03×10^6	95	0.50
15	0.72×10^6	75	0.90

cular structures (even in small quantity) is responsible mainly for the difficulties encountered in the characterization of polysaccharides by light scattering and SEC.

Unambigous interpretation of MALLS data demands precise determination of Rg and M_w at each retention volume in SEC. Concerning the difficulties for characterizing the solution behavior of high M_w rigid polysaccharides, it is obvious that useful data would result of adding an on-line capillary viscometer detector as proposed by Ouano [19] and Kuo et al. [20] and improved by Lesec et al. [21]. SEC-viscometry combination would be particularly useful for the differentiation between microgels/aggregates and high M_w polymer fractions. More precisely, the combination of SEC with MALLS and viscosity detectors [18] would lead to interesting conclusions from analysis of the M_w dependence of both the radius of gyration (as deduced from light scattering) and the viscosity radius $R\eta$, i.e., the radius deduced from Einstein's law for hard spheres. From the value of the ratio $Rg/R\eta$ one can expect interesting and useful information on the branching density and the shape of the microgels. However, it should be mentioned that the interpretation of the vis-

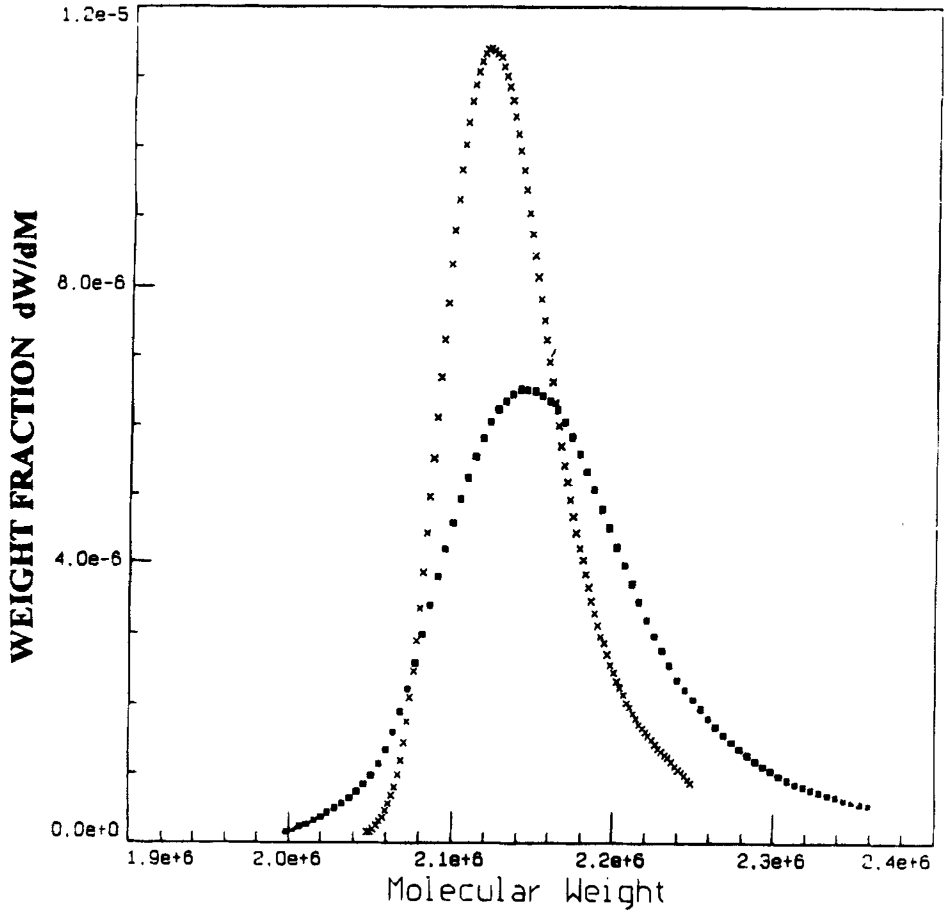

FIGURE 9 Differential molecular weight distribution of a schizophyllan sample in the native state (■ ■) and after extensive ultrafiltration (x x).

cometric detector can be complicated by the dependence of the viscosity with shear rate. This effect can lead to an overestimation of the elution volume, particularly in the case of high M_w rigid polymers [22].

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