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Short communication

# On-line direct determination of the second virial coefficient of a natural polysaccharide using size-exclusion chromatography and multi-angle laser light scattering

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**Abstract**

By combining a size-exclusion chromatographic (SEC) separation and an on-line multi-angle light scattering (MALLS) analysis, we have elaborated an original methodology permitting on-line direct determination of the second virial coefficient of molar mass fractions of polydisperse polysaccharides. By assimilating the SEC–MALLS data to a batch mode acquisition, we have obtained on-line the complete Zimm plot of the eluted fractions, leading to knowledge of their weight-average molar mass $M_w$, radius of gyration $r_g$ and second virial coefficient $A_2$. Our methodology was successfully applied to a iota carrageenan sample in LiCl 100 m*M*, EDTA 1 g/l. 

*Keywords:* Zimm plots; Second virial coefficient; Polysaccharides

## 1. Introduction

Polysaccharides are an important group of water-soluble polymers and are used in many different applications, for example food additives and drug formulations. One effect of the increased interest is a growing need for information about the solution properties of polysaccharides. This knowledge is essential in order to predict their behavior in processes and products, but the characterization of polysaccharides from natural origin is often a difficult task.

Light scattering is one of the few standardless methods available for the determination of the molar mass and shape of polymers [1–4]. Applied to unseparated polymers, these measurements produce the weight-average molar mass $M_w$, and the corresponding average radius of gyration $r_g$, together with the second virial coefficient $A_2$, which characterizes polymer–solvent interactions. These are average values with poor significance in the case of polysaccharides with large polydispersity.

As a consequence it is of primary importance to separate these compounds in a reproducible way and to characterize the fractions obtained. Great efforts have been made in recent years to isolate the various

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fractions constituting the samples. A number of techniques has been used, notably gel permeation [5,6], hydrophobic affinity [7], ion-exchange chromatography [8,9], and precipitation [10]. Most of these techniques are quite arduous and time-consuming, and very few of them offer the possibility to analyze the fractions at the same time. The combination of light scattering and HPSEC (more commonly called size-exclusion chromatography, SEC) has been successfully applied to the separation and analysis of natural polysaccharides [9,11–15]. By combining SEC and multi-angle laser light scattering (MALLS) detection, weight, number and *z*-average values for both mass and size may be obtained for most samples. Moreover, chromatographic analysis permits access to the molar mass distribution and polydispersity of the samples [16]. In spite of the good results obtained, one major problem still remains: no information about the polymer fractions–solvent interaction (i.e. the $A_2$ coefficient) can be obtained by this method.

In the present article, we propose a methodology to separate and characterize the molar mass fractions of a polydisperse polysaccharide sample at the same time. By calculating on-line Zimm plots on a SEC-separated sample, one can obtain $M_w$, $r_g$ and $A_2$ of the sub-populations of chains constituting the sample. This approach could be useful in providing greater insight into the characterization of the various species that constitute polysaccharides without carrying out elaborate fractionation procedures prior to analysis. Our procedure has been applied to iota (ι)-carrageenans, which are polydisperse polysaccharides extracted from seaweed.

## 2. Methodological background

### *2.1. Batch mode*

By working in a batch mode with a MALLS detector, one can directly obtain $M_w$, $r_g$, and $A_2$ of an unseparated polymer by computing a classical Zimm plot from light scattering data collected at various angles ($\theta$) for each polymer concentration ($c$). Practically, the scattering intensity is converted into an excess Rayleigh ratio, $R_\theta$, and the quantity $Kc/R_\theta$ is plotted versus $\sin^2\theta/2 + Sc$, with $K$ being an optical constant and $S$ a stretch factor selected to obtain a well-defined plot. The small angle data are extrapolated to 0° for each concentration, and the data at each angle are extrapolated to zero concentration. The common intercept of the two extrapolated curves yields the reciprocal $M_w$. The slope of the $c=0$ curve near $\sin^2\theta/2 = 0$ yields $r_g$, while the slope of the $\sin^2\theta/2 = 0$ curve near $c=0$ yields $A_2$. It is clear that, with this method, the molecular distribution is averaged. In the case of natural polysaccharides, the polydispersity of the samples might obscure the analysis of the scattering data.

### *2.2. Conventional SEC–MALLS analysis*

When coupling a SEC system to a MALLS detector, the sample analysis only requires a single injection. Data are collected in a computer file as slices, which are the computed number of data points collected per detector, based on flow-rate, collection duration and collection interval (as an example, under our experimental conditions, a file corresponding to a single injection displays 1599 slices). $M_w$ and $r_g$ (determined from the intercept and the slope of a fitting curve corresponding to constant concentration, respectively) of each slice constituting the area of the peak selected are calculated by the software. These results are then averaged to obtain $M_w$ and $r_g$ of the chromatographic peak selected. The polydispersity of the sample can also be evaluated from the ratio of the weight-average molar mass to the number-average molar mass, $M_w/M_n$. But a conventional on-line light scattering analysis of the eluted fractions after a SEC separation does not give access to the second virial coefficient of the fractions. Since the concentration in each slice is not multiple, information about $A_2$ is not available.

## 3. Procedure

By combining the two possibilities offered by a MALLS detector, i.e. conventional SEC–MALLS analysis and batch mode analysis, we have elaborated a methodology permitting us to obtain on-line the $M_w$, $r_g$ and $A_2$ values of the eluted fractions of a polydisperse polysaccharide sample in a given solvent. To do this, we sequentially injected the initial preparation of the sample and three different dilutions in ascending order of concentration into the

SEC–MALLS–refractive index (RI) detection system. The chromatographic data were collected in one single software run file and then processed as one single chromatogram having four peaks, one for each sample injection. After setting an appropriate baseline on the whole chromatogram (corresponding to the four successive sample injections), the volume intervals to analyze each of the four peaks were selected. These intervals are constituted of a determined number of slices, centered on the molar mass interval of the peak one wants to characterize. Under our experimental conditions, we chose intervals of 11 slices, corresponding to an elution volume fraction of 0.2 ml. The intervals selected can be considered as four increasing concentrations of the same molar mass fraction of the polymer. These concentrations are calculated by the refractometer and reported in the software. The concentration values are then manually transferred as sample concentrations into the part of the software that performs data analyses in the batch mode. Then the Zimm plot corresponding to this molar mass fraction can be calculated by the software and give $M_w$, $r_g$ and $A_2$. From a single file, corresponding to four successive injections, Zimm plots corresponding to several different intervals of the eluted peaks can be obtained.

An analyzable molar mass interval (giving accurate results) must be determined. Two conditions were necessary to obtain accurate Zimm plots of the fractions. The first was that the correspondence between elution volume, molar mass and radius of gyration had to be the same for all the concentrations injected. The second was that the MALLS and RI signals had be sufficiently high to permit accurate ASTRA calculations. This could be verified by the high degree of agreement between the results for $M_w$ and $r_g$ obtained by the software after a conventional SEC–MALLS analysis and after a batch mode analysis, i.e. when assimilating the results to a batch mode acquisition.

## 4. Experimental

### 4.1. Reagents

The solvent used during the experiments was a solution of LiCl 100 m*M* (RP Normapur, Prolabo, France), ethylenediaminetetraacetic acid (EDTA) 1 g/l (analytical-reagent grade, Merck, Darmstadt, Germany), in ultrapure water (Milli-Q, Millipore, Bedford, MA, USA). It was filtered through a 0.1 μm filter (Millex HV, Millipore), degassed and the pH was adjusted to 6.5 with 1 *M* HCl or 1 *M* NaOH (RP Normapur).

ι-Carrageenan was a commercial sample supplied by SKW (France). κ-Type impurity was less than ca. 5% as determined by NMR. The majority of the sample studied was in the $K^+$ form and was used without any further purification. A conventional SEC–MALLS analysis of the sample in LiCl 100 m*M*, EDTA 1 g/l resulted in an $M_w$ of 605 200 g/mol, a polydispersity index $M_w/M_n$ of 1.363, and an $r_g$ of 66 nm.

### 4.2. Preparation of dilute polysaccharide solutions

An initial solution of ι-carrageenan and its dilutions were prepared by stirring at 80°C for 30 min. The concentration in the initial preparation was set to 1 mg/ml. The solutions were then cooled to room temperature (25°C) and filtered through a 0.45 μm filter (Millex HV, Millipore). All the samples studied had the same storage period of 24 h at 25°C in complete darkness.

### 4.3. Apparatus

The SEC equipment consisted of an intelligent pump (Flom, USA) with an on-line degasser (Gastorr102, Flom) and a 0.1 μm on-line filter (Durapore, Millipore). SEC was performed with a Shodex (Showa Denko, Japan) OH-Pak-SB-G guard column followed by Shodex OH-Pak columns B-806/HQ, B-805/HQ, and B804/HQ in series, placed in a thermostated oven (CrocoCil, France), the temperature of which was set to 60°C to preclude any aggregate formation. The flow-rate was set at 0.6 ml/min during all the experiments and the injected volume was 250 μl. The whole system was placed in a temperature-controlled room (25°C).

Two detectors were used: a DAWN DSP (Wyatt Technology, USA) MALLS detector, equipped with a F2 flow cell and a He–Ne laser light source ($\lambda = 632.8$ nm), and a refractometer (RI ERC A-7515, Japan) operating at 632.8 nm. Data collection and processing were under the control of a personal

computer driven by the Wyatt Technology ASTRA program. The light scattering signal was detected simultaneously at 11 scattering angles, $\theta$, ranging from 35 to 132°.

The differential refractive index increment d$n$/d$c$ of ι-carrageenan in LiCl 100 m$M$, EDTA 1 g/l was determined using an interferometric refractometer Wyatt/Optilab 903 (Wyatt Technology) operating at 632.8 nm. It was averaged to 0.115 ml/g (with a standard deviation of 0.0005) and was assumed to be constant over the sample elution.

## 5. Application of the procedure to ι-carrageenans

The capability of our procedure was tested on a ι-carrageenan sample in LiCl 100 m$M$, EDTA 1 mg/ml. ι-Carrageenans are anionic polysaccharides extracted from seaweed. They are widely used in food for their thickening and gelling properties. Because of their widespread use, there is a growing need of information about their behavior in solution. Their light scattering analysis is generally carried out after a purification and/or a fractionation step. In the case of ι-carrageenan, this step is problematic [6,14] and often leads to unreproducible and/or uninterpretable results. This was attributed to the possible existence of different conformations and/or association of chains in the fractions [6], possibly due to the presence of a limited amount of low-molar-mass chains obscured by a greater proportion of large molecules [14], without being clearly elucidated. The application of our methodology could be useful in providing a greater insight into the behavior of these polysaccharides in solution.

The SEC separation of carrageenans is well described in the literature [14,15]. The solvent employed for our experiments was chosen based on the results of previous SEC–MALLS analyses of carrageenans: $Li^+$ ions are reported to prevent or reduce the tendency of carrageenan chains to associate [14,17]. Under our experimental conditions the polysaccharide chains present a random coil shape; the chromatogram displays a single peak.

Fig. 1 shows the SEC–MALLS chromatograms corresponding to four successive injections of ι-carrageenan solutions in ascending order of concentration. Four molar mass fractions, a, b, c and d, were studied within the analyzable molar mass interval and include the upper (fraction a) and the lower (fraction d) limits. Fig. 2 displays the intervals corresponding to these molar mass fractions in the first chromatographic peak. As an example, Fig. 1 shows the volume intervals selected on each peak for the analysis of the 870 000 g/mol molar mass fraction (fraction b). With our methodology the Zimm plot corresponding to this molar mass fraction can be obtained (Fig. 3) and gives $M_w$, $r_g$ and $A_2$. The other eluted fractions were processed in the

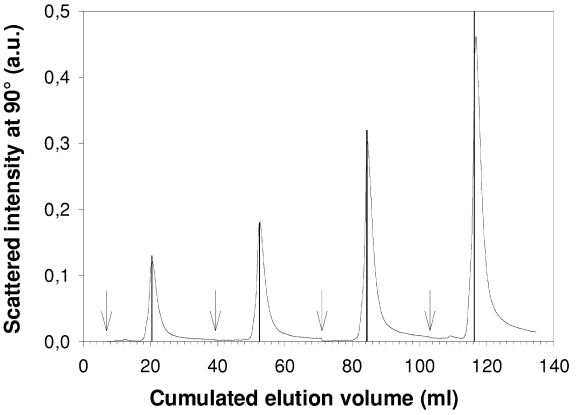


Fig. 1. SEC–MALLS chromatograms of increasing amounts of ι-carrageenan in LiCl 100 m$M$, EDTA 1 g/l and intervals of peaks selected for the Zimm plot of the 870 000 g/mol molar mass fraction (fraction b). The beginning of each acquisition is indicated by a vertical arrow.

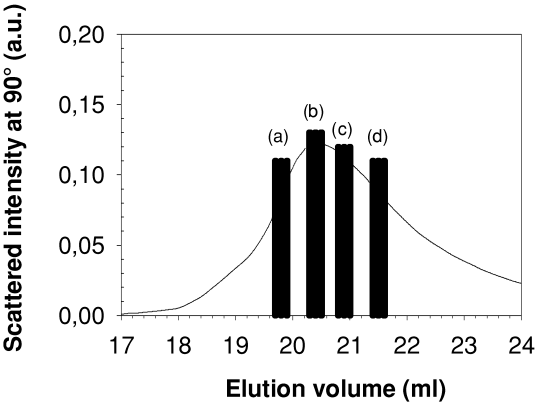


Fig. 2. Detail of the intervals corresponding to the four molar mass fractions studied (a, b, c and d) in the first chromatographic peak.

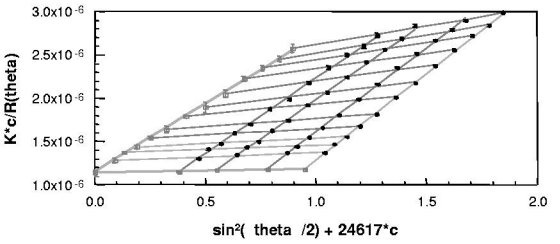


Fig. 3. Zimm plot of the 870 000 g/mol molar mass fraction (fraction b) of ι-carrageenan in LiCl 100 m*M*, EDTA 1 g/l.

same way; the results of the Zimm plot calculations are presented in Table 1. They show a concomitant decrease of $A_2$ and $r_g$ while the $M_w$ of the fractions decreases. To evaluate the precision of the determination of $A_2$, the ι-carrageenan sample was analyzed three times according to our methodology. A one-way analysis of variance (ANOVA) followed by a multiple range test was performed in order to compare the mean values of $A_2$ for the four different $M_w$ fractions studied. The statistical analysis showed that the observed changes in $A_2$ are significant at the 95% confidence level. The positive $A_2$ values measured at high $M_w$ reflect a good solvent quality. In the case of the lowest analyzable molar mass fractions, $A_2$ becomes significantly negative, indicating that near theta solvent conditions prevail. These results suggest that a significant change of the chains–solvent interaction accompanies the reduction of the coil dimensions for the lowest-molar-mass fractions and could explain some of the uninterpretable light scattering results reported for ι-carrageenans [6,14]. This modification of the ι-carrageenan chains–solvent interaction according to molar mass will be the subject of a more detailed study in order to provide an answer to this assumption. This will be reported in a subsequent paper.

Therefore, the original approach of the SEC–MALLS technique developed here permits us to obtain information that cannot be obtained by a conventional SEC–MALLS analysis. The use of this methodology has proved valuable in permitting on-line determination of the $A_2$ of polysaccharide fractions previously separated by SEC. Its application to ι-carrageenans has given interesting results that will be investigated further in our laboratory.

Table 1
$M_w$, $r_g$ and $A_2$ values of ι-carrageenan fractions corresponding to the upper limit (a), the middle (b and c) and the lower limit (d) of the analyzable molar mass interval, obtained by Zimm plot calculations ($n=3$)

| Eluted interval | Replicate | $M_w$ (g/mol) | $r_g$ (nm) | $A_2$ (mol ml/g$^2$) |
|---|---|---|---|---|
| a | 1 | 1 183 200 | 93 | $11.9\cdot10^{-4}$ |
| | 2 | 1 203 000 | 96 | $14.1\cdot10^{-4}$ |
| | 3 | 1 212 600 | 99 | $16.3\cdot10^{-4}$ |
| b | 1 | 864 000 | 74 | $4.9\cdot10^{-4}$ |
| | 2 | 873 400 | 76 | $6.4\cdot10^{-4}$ |
| | 3 | 882 000 | 77 | $7.8\cdot10^{-4}$ |
| c | 1 | 645 200 | 62 | $-7.9\cdot10^{-4}$ |
| | 2 | 653 900 | 64 | $-9.5\cdot10^{-4}$ |
| | 3 | 665 300 | 66 | $-5.8\cdot10^{-4}$ |
| d | 1 | 436 700 | 51 | $-14.1\cdot10^{-4}$ |
| | 2 | 454 400 | 53 | $-11.6\cdot10^{-4}$ |
| | 3 | 461 200 | 53 | $-10\cdot10^{-4}$ |

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