

Alginates, chitosanes and xanthans

Characterisation of food ingredients by GPC/SEC with triple detection

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ABSTRACT: Modern foodstuffs often contain a variety of ingredients used to optimise specific properties of the products such as viscosity or stability. Some of these ingredients come from natural sources. As an example alginates are made from brown algae, chitosans are made from shellfish and xanthans are made from sugar with the help of microorganisms. While alginates and xanthans are directly added to the food, chitosans are often used separately and are reported to reduce the amount of fat that is absorbed by the human body and therefore help reduce the weight of the person using them. The performance characteristics of all these food ingredients will depend strongly on their molecular weight and structure so determination of an accurate molecular weight distribution is paramount. In addition, there may occasionally be suspicions that the low molecular weight fractions of these ingredients could be harmful (1), so a powerful analytical technique must be available to make sure that those potentially dangerous parts of the ingredients have been removed before they are added to any food. Using the technique of GPC/SEC with triple detection, a comprehensive characterisation of food ingredients can be achieved. Due to the large molecular size of these samples Low Angle Light Scattering Detector (LALS) is an especially important tool in the system for the accurate analysis of molecular weight and molecular structure.

KEYWORDS: Gel Permeation Chromatography, Size Exclusion Chromatography, GPC, SEC, Light Scattering, Low Angle Light Scattering, Viscometer, Alginates, Chitosans, Xanthans, Gum Arabic, Carrageenans.

INTRODUCTION

Many of these food ingredients are biopolysaccharides such as dextrans and starches. Some of these samples can be analysed using standard conditions; some are difficult and require special columns, instruments and methods. The very difficult to analyse samples are those with extremely high molecular weight and highly branched structures. To analyse such samples accurately, powerful instruments must be used. For those samples the Low Angle Light Scattering Detector (LALS) is particularly important (2, 3). Very often molecular weight is not the only parameter of interest to fully characterise a biopolymer sample. GPC/SEC with triple detection can also determine the structure of a biopolysaccharide sample using the viscosity detector. Structure in this case is related to branching structure and hydrodynamic size of the biomolecules (4, 5).

LIGHT SCATTERING

For molecular weight determination SEC/GPC is combined with a so-called 'static' light scattering detector. The intensity of scattered light depends on the molecular mass of molecules in the analyte solution. The relationship is given by the Zimm equation:

$$\frac{K'c}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2c + \Lambda \quad \text{where} \quad K' = \left(\frac{dn}{dc} \right)^2 (1 + \cos^2 \theta) \times \text{const}$$

(Zimm Equation)

R_θ is the Rayleigh factor (function of scattered light intensity), c is the concentration of the sample in the solvent, θ is the scattering angle, M_w is the weight average molecular weight of the sample, dn/dc is the refractive index increment of the polymer solution, and A_2 is the second virial coefficient which corrects for interaction of polymer molecules with each other

and can be calculated from the concentration dependence of the light scattering signal, $P(\theta)$ is the particle scattering function which is a measure of the angular dissymmetry of the scattered light and is related to the size and the angle at which the scattering is determined.

At 0° scattering angle the equation simplifies to: $K'c/R_\theta = 1/M_w$. Of course, it is impossible to measure at 0° due to the incident light beam. The actual measurement has to be taken at a different angle. Where the molecules are larger than approximately 10-15 nm, intramolecular interference leads to angular dependence of the scattering intensity: $P(\theta) \neq 1$. The LALS approach – measuring at 7° – gives a very accurate value as it is close to the true value at 0° . The error at normal molecular weights is negligible and even at extremely high molecular weights is still acceptable. For example, measuring at 7° rather than 0° leads to a deviation from the true result of only about 2.5 percent for 10,000,000 Da polystyrene molecules.

The other approach is multi angle light Scattering (MALS). A MALS detector incorporates several photo detectors surrounding the sample cell at various angles. The intensities measured at different angles are then extrapolated to give an estimated 0° value. Commercial MALS detectors do not have as low scattering angles as LALS detectors and often the lowest angle useable is 30° to 45° . Measuring a 10,000,000 Da Polystyrene at 30° would lead to an underestimation of 30 percent of the M_w . This shows how critical fitting and extrapolating the data is for a MALS system. An additional difficulty arises from different functions for fitting the data which are all based on different scientific models and lead to different results. Therefore the LALS detector is preferable as it directly measures the molecular weight and requires no user pre-knowledge.

ALGINATES

Alginates are mainly made of brown algae; they are widely used as gelling agents. More and more companies produce or process alginates and research institutes investigate alginates and are interested in a comprehensive characterisation regarding molecular weight distribution and molecular

structure of alginate samples. A suitable technique therefore is GPC/SEC with triple detection. This technique separates the alginate molecules by size before molecular weight and molecular structure are determined at each point of the distribution.

A typical triple detection chromatogram is shown in Figure 1. It is an alginate sample with an average molecular weight (M_w) of 993,000 Da and a mass average intrinsic viscosity ($[\eta]_w$) of 2,810 ml/g. An average hydrodynamic radius ($R_{h,w}$) of 74.4 nm can be calculated using M_w and $[\eta]_w$.

Compared to an alginate sample that was treated in an autoclave before analysis it can be observed that the autoclave sample has a lower molecular weight ($M_w = 105,000$ Da) and a lower intrinsic viscosity ($[\eta]_w = 415$ ml/g, $R_{h,w} = 17.9$ nm) than the untreated sample. Obviously the autoclave treatment leads to a degeneration of the alginate sample. Figure 2 shows the differential and cumulative molecular weight distributions of both alginate samples.

The relatively high intrinsic viscosity values found for both alginate samples prove that alginates have an open, stiff chain structure in solution. As a comparison: a dextran sample (branched polysaccharide, coil structure) has an intrinsic viscosity of only 34 ml/g at a molecular weight of 150,000 Da. The hydrodynamic radius of the dextran sample is 9.16 nm.

The open, stretched structure of alginates is also confirmed by the Mark-Houwink plot (Figure 3) for both alginate samples. The Mark-Houwink plot is the central structure plot used in GPC/SEC. Log intrinsic viscosity ($[\eta]$) is plotted versus log molecular weight (M). The a -value is the slope of the straight line and log k is the axis intercept.

$$\log [\eta] = \log k + a \times \log M \quad (\text{Mark-Houwink-Equation})$$

For a polymer or biopolymer sample with a linear chain structure without branches an a -value of 0.6 to 0.8 results. Branched samples show lower a -values while stiff chain, stretched samples show higher a -values.

For both alginate samples a -values of 0.8 to 1.1 are found. These values confirm the open, stiff structure of the samples. Due to this unusual molecular structure of the alginate samples a simple molecular weight comparison versus Dextran or Pullulan Standards will end up with completely wrong numbers. Dextrans and Pullulans are much smaller molecules in solution at a certain molecular weight than alginates. Therefore the retention time in GPC/SEC is longer for dextran and pullulan samples than for alginates. The relative result for molecular weight of any alginate sample therefore will be too high. Only the Low Angle Light Scattering Detector (LALS) can accurately measure molecular weight of alginates without extrapolation.

CHITOSANS

Chitosans are used as food excipients. It is assumed that they absorb large amounts of fat in the human body which should finally lead to a loss of weight. These assumptions have not been proven yet in a scientific way (6). Chitosans are also often added to cosmetics. To be able to analyse Chitosans with GPC/SEC it is important to know that Chitosans are only

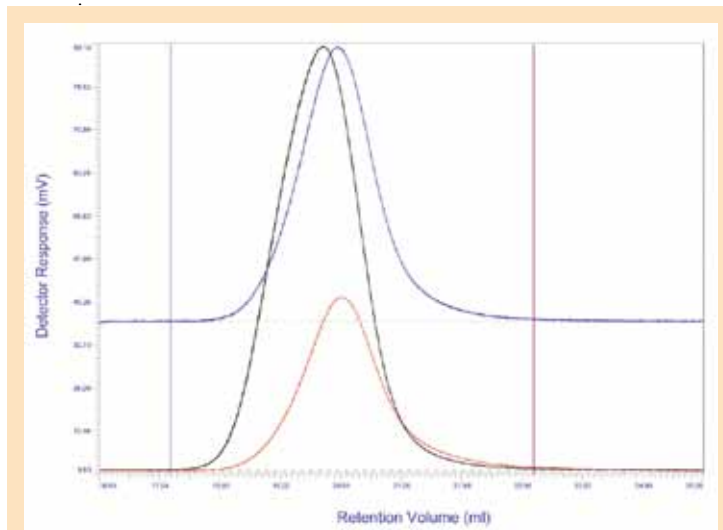


Figure 1. Triple detection chromatogram of an alginate sample. red: refractive index detector, blue: viscosity detector, black: Low Angle Light Scattering Detector.

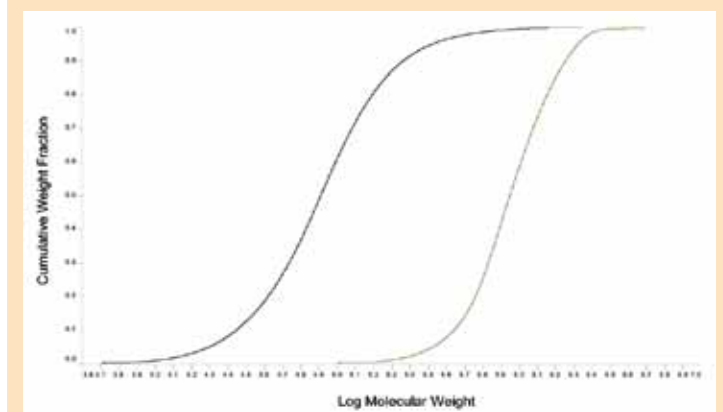
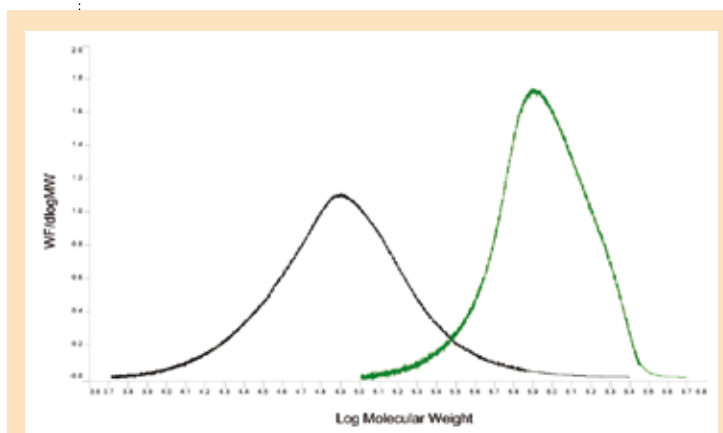


Figure 2. Differential (top) and cumulative (bottom) molecular weight distributions of 2 alginate samples. black: autoclave sample, green: untreated sample.

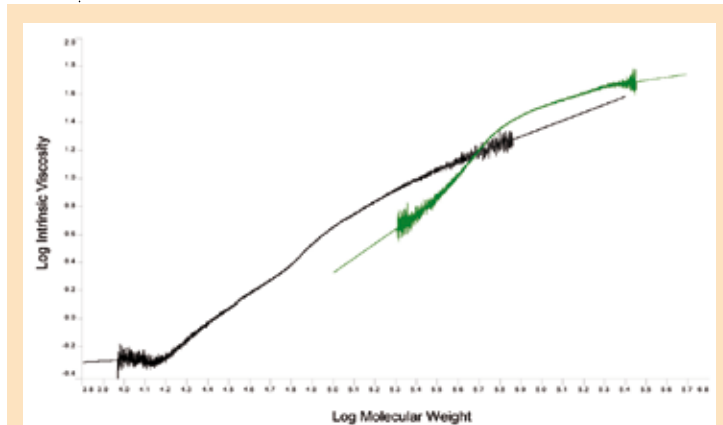


Figure 3. Mark-Houwink-Plots of 2 alginate samples. Black: autoclave sample, green: untreated sample.

soluble at a pH of less than 6.3. Therefore the solvent used for the GPC/SEC analysis must be acidic. To achieve this sometimes trifluoro acetic acid is added to the aqueous buffer system. It

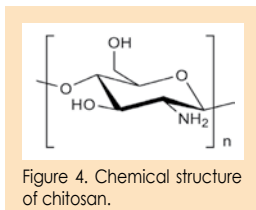


Figure 4. Chemical structure of chitosan.

is also important to know that the chitosan samples need a long time for dissolving; this can be up to 18 hours. For some samples elevated temperatures are needed (40°C) to dissolve them completely. The user must be careful not to degrade the samples during the sample preparation procedure. Before injection the chitosan samples should be filtered using e.g. a 0.45 μm nylon filter. ViscoGel GPC/SEC Columns proved to be very suitable columns for the analysis of chitosans. These columns can separate chitosan samples up to a molecular weight of several million daltons. An overlay of the refractive index chromatograms and the Mark-Houwink-Plots of 3 chitosan samples are shown in Figure 5. The three chitosan samples can easily be separated by their molecular weight distributions. There is no significant difference observed in the structures of the three samples. Table 1 shows the numerical results from the GPC/SEC experiment: molecular weight, intrinsic viscosity and hydrodynamic radius. The hydrodynamic radius is measured both with the light scattering detector and the viscosity detector. A radius below 10 nm can not be measured by static light scattering on its own as the scattering is isotropic.

XANTHANS

Xanthan is a natural gelling agent. It is produced by xanthomonas bacteria using sugar-containing substrates and labelled as food additive E415. Xanthan molecules are very large and therefore found at the upper end of the GPC/SEC application spectrum. The hydrodynamic radius of xanthan molecules sometimes exceeds 100 nm so separation columns with a very large pore size must be used for GPC/SEC analysis of these biomolecules. A triple detection chromatogram of a xanthan sample is shown in Figure 6. The sample has a relatively narrow distribution with a polydispersity (M_w/M_n) of 1.1. The average molecular weight of the xanthan sample is 7,741,000 Da, it has an intrinsic viscosity of 2,294 ml/g. The hydrodynamic radius is determined as 140.7 nm. In Figure 7 the molecular weight (black line) and hydrodynamic radius (green line) of the xanthan sample is plotted versus retention volume. The molecular weight plot shows that there are some weak interactions between the xanthan sample and the packing material of the GPC/SEC column. The interactions between the xanthan sample and the packing material of the GPC/SEC column can be seen by the increasing molecular weight curve at large retention volumes. This effect is rather small and has only minor impact on the GPC/SEC results.

FURTHER BIOPOLYMER APPLICATIONS

Besides the substances discussed above, there are many more samples from the food additive area that can be analysed by GPC/SEC with triple detection. Due to the fact

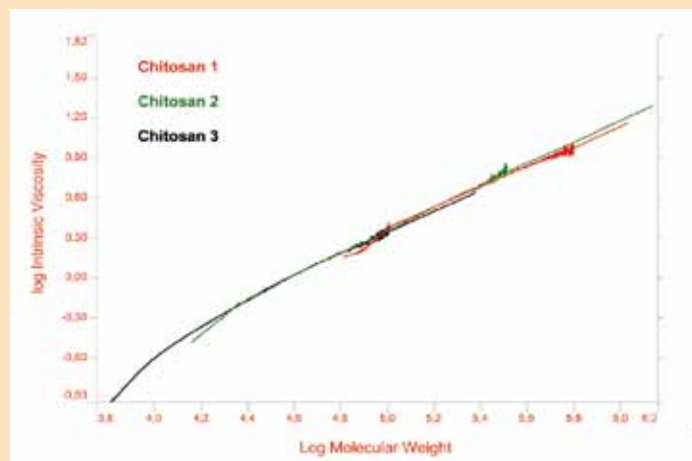
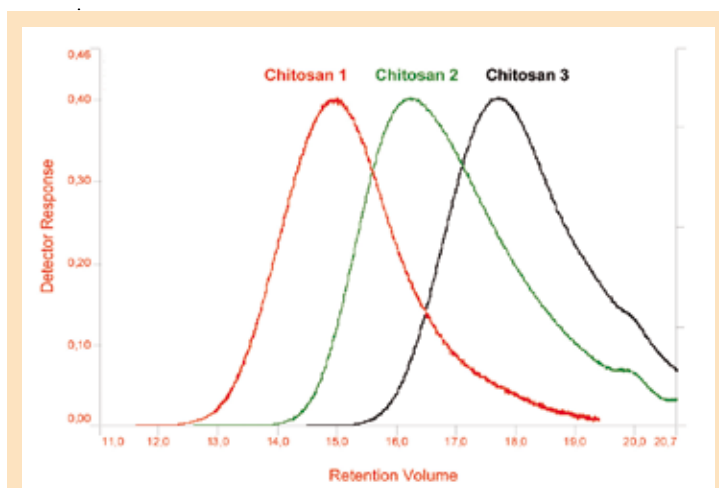


Figure 5. Overlay of refractive index chromatograms (top) and Mark-Houwink-Plots (bottom) of 3 chitosan samples.

	Chitosan 1	Chitosan 2	Chitosan 3
M_w in g/mol	19,800	78,700	201,700
$[\eta]$ in ml/g	46.5	178	361.2
R_h in nm	5.2	12.1	22.9

Table 1. Numerical results for three chitosan samples.

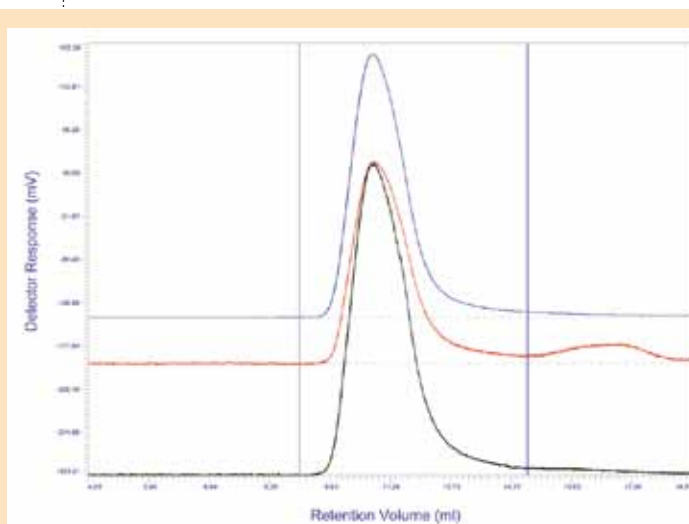


Figure 6. Triple detection chromatogram of a xanthan-sample, red: refractive index detector, blue: viscosity detector, black: Low Angle Light Scattering Detector.

that various biopolysaccharides may have completely different structures it is important that the GPC/SEC-system is optimised for each kind of sample. One of the important parameters is the buffer system that is used as eluent and the pH of the aqueous phase. Also the separation range of the GPC/SEC columns must fit to the molecular weight range of each sample. Temperature can be an important parameter

for special samples; e.g. for carrageenans it is important to run the columns at an elevated temperature of app. 60°C. Otherwise they will aggregate at lower temperatures and the resulting molecular weight will be too high (7).

If a UV-detector is used additionally then UV-active components can be determined and for some kind of samples the chemical composition can be analysed. One example is the determination of protein content in Gum Arabic, another natural gelling agent such as xanthan.

SUMMARY

The powerful technique of GPC/SEC with triple detection (Low Angle Light Scattering, Viscosity Detection and Refractive Index Detection) can be used for the comprehensive characterisation of complex food ingredients and additives based on biopolysaccharides. Besides the absolute molecular weight distributions of these samples their molecular structure and branching frequency can be determined in solution. The Low Angle Light Scattering detector particularly is an important tool for the analysis of biopolysaccharides. It is the only detector available that can directly measure molecular weight of a sample without any need of data extrapolation. The viscosity detector measures the intrinsic viscosity of the sample that is needed to create the Mark-Houwink structural plot and determine hydrodynamic radius and degree of branching for each sample without any limitation in size.

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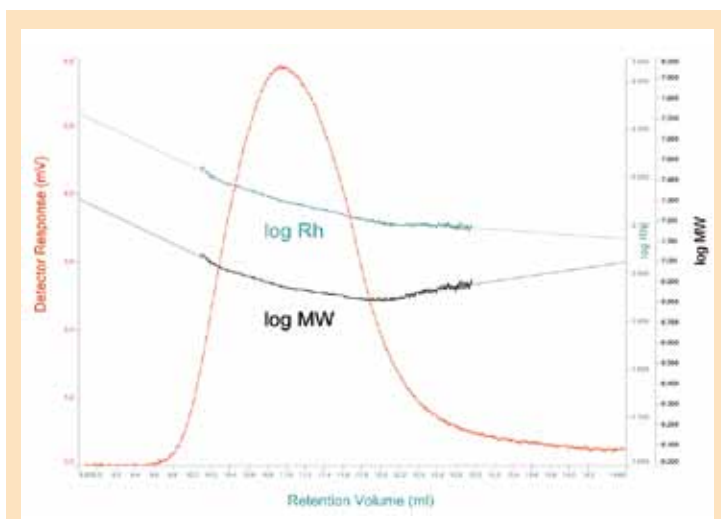


Figure 7. GPC/SEC results for a xanthan-sample: red: baseline corrected refractive index chromatogram; black: log Mw versus retention volume; green: log Rh (hydrodynamic radius) versus retention volume.

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Calcium caprylate and magnesium caprylate are salts of a medium-chain fatty acid. They are insoluble in water but would be expected to dissociate in the acidic environment of the stomach and the bioavailability of the respective cations is expected to be similar to that of other, soluble sources of these cations. There were no toxicological data on calcium caprylate or magnesium caprylate in the dossiers. Few toxicological data are available on caprylic acid while several studies have been performed with triglycerides containing different percentages of caprylates. Caprylic acid exhibited no mutagenic activity in microbial mutation assays. The Panel concluded that the molecule has no structural alert for genotoxicity and that the data available do not raise concern with respect to genotoxicity. The Panel considered whether studies in animals with triglycerides could provide a basis for the assessment of caprylic acid according to the percentage of caprylic acid present in the triglycerides tested. A 91-day study performed on the triglyceride caprenin in mice allowed the derivation of a No-Observed-Adverse-Effect-Level (NOAEL) equal to 3036 and 3358mg/kg bw/day of caprylate for male and female mice respectively. The Panel identified a NOAEL from the 2-year NTP (1994) study on rats exposed to the triglyceride tricaprylin at 2.5mL/kg bw/day, equivalent to approximately 2400mg/kg

bw/day of tricaprylin and 1900mg/kg bw/day of caprylate. The NTP study (1994) appears to be the more relevant for risk assessment of caprylic acid than the 91-day study in mice. Using the NOAEL of 1900mg/kg bw/day for caprylate would result in a Margin Of Safety of 12, in relation to the estimated exposure from the proposed uses, which the Panel would consider inadequate. However, the Panel noted that the effects in the forestomach observed in the NTP study (which were the basis for the NOAEL) might be specific for tricaprylin in rats and should not therefore be extrapolated to caprylic acid. The proposed use levels would result in exposures to caprylic acid around 10- to 30-fold higher than the estimated exposure to caprylate from the diet. The Panel considers that since the exposures from the proposed use levels for calcium caprylate and magnesium caprylate are not comparable to the estimated dietary exposures for caprylic acid, and would result from a single bolus dose, it is not possible to assume that there would be sufficient capacity to handle this load of caprylic acid using normal metabolic pathways. However, the Panel notes that the nutrients are only 7-12% by weight of the caprylate, so if calcium and magnesium were administered at the UL, exposure to caprylic acid from the sources would be significant, both in absolute weight and in calorific terms. The Panel concludes that there are insufficient toxicological data on caprylic

acid itself to allow conclusions on the safety of the proposed use and use levels of calcium caprylate and magnesium caprylate. The Panel notes that read across from the tricaprylin data would result in inadequate safety margins but concludes that such a read across is inappropriate since the forestomach effect may result from tricaprylin and not caprylic acid. Nevertheless, the caprylin data could indicate possible concerns about caprylic acid. Also, the Panel highlights the paucity of toxicological data on caprylic acid, and considers that long term toxicity data on caprylic acid would be needed for an adequate assessment. The Panel concludes that the amounts of caprylic acid from the proposed use levels are far greater than dietary exposures and it is therefore not possible to base a safety evaluation on the normal metabolism of dietary caprylic acid. The Panel therefore concludes that the safety of calcium caprylate and magnesium caprylate, as sources of calcium and magnesium respectively, at the proposed use levels cannot be established based on the available information.

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