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Characterisation of chitosan molecular weight distribution by multi-detection asymmetric flow-field flow fractionation (AF4) and SEC

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1. Introduction

The multiple functionality of chitosan with more than 200 current and potential applications across different scientific areas such as biomedicine, pharmacy, biology, environment, agriculture and foods as well as its natural and sustainable character, has greatly increased the commercial interest and use of this biopolymer. Many of its material and biological properties are intimately related or linked to the molecular weight (MW) of the polymer. Examples are its biodegradability, its potential to interact with the mucosal surfaces, its hemostatic, antimicrobial and anticholesterolemic properties as well as the material properties derived from its polymeric nature such as formation of hydrogels, supramolecular particles (complexes), nanoparticles, and scaffold materials among others [1-10]. Chitosan-based biomaterials have also become well-established drug, gene and protein delivery platforms, particularly for biologics (e.g. insulin, genes, etc.) across mucosal epithelial barriers [11-14]. Therefore, the accurate determination and full characterisation of chitosans physicochemical properties is becoming ever more necessary. MW is particularly important because it influences the functionality and bioactivity and, as for most other polysaccharides, varies greatly depending on the biological source (e.g., exoskeletons of crustaceans and insects, cell walls of fungi, fish scales), the season of harvest and the process of isolation of chitin and the deacetylation into chitosan. Properties such as the mechanical properties of hydrogels, pore size of membranes and scaffolds, the particle size of nanoparticles and nanocapsules [15], the effect on the permeability of epithelial cells[16], the intracellular delivery of genes[17] and the antimicrobial properties [9], among other, are also influenced by the MW of the chitosan. Both MW and degree of acetylation (DA) are the parameters that ultimately determine the performance of

chitosan in biotechnology, food, pharmaceutical, materials and biomedical applications. Robust analytical characterisation methods are essential to guarantee reliability and reproducibility of the polymer chemical, physicochemical material and biological properties. The full characterisation of chitosan is also very important for the continued research on "second" generation chitosans and the future development of "third" generation polymers that can ensure consistency in their performance.

The main techniques used to determine the molecular weight of chitosan are similar to those used for any polymer, namely viscometry, light scattering, high performance size exclusion chromatography (GPC or SEC), osmometry, and sedimentation equilibrium by centrifugation [18]. More recently, a method using atomic force microscopy has also been reported [19]. All of these techniques require the complete dissolution of the polymer chains by the solvent (normally an acidic aqueous solution with sufficient ionic strength provided by a suitable salt). Coupling of SEC to multi-detectors, namely differential refractive index (RI) and multi-angle light scattering (MALS), is a powerful strategy to determining the molar mass and its distribution while avoiding the need of calibrating with standards of narrow MW distribution such as pullulans. However, inaccuracies can arise when using such methods due to the presence of intermolecular aggregates present even at low polymer concentrations (~1 mg/mL), as has been highlighted previously by several research works. The mechanism of self-association continues being widely discussed in this context [20].

Asymmetric flow field flow fractionation (AF-FFF or AF4) is a separation technique using the principle that laminar flow in a long thin channel has a parabolic profile and that polymers and particles driven by a force perpendicular to this flow will, depending on their diffusion coefficients, arrange themselves with different mean layer thicknesses from the channel bottom

so they are transported with different velocities through the channel. This results in a separation according to size from small to large in which the size resolution can be controlled by the cross flow force [21].

Few recent studies have reported the use of AF4 to characterise the MW distribution of chitosan (Table 1) [22-24]. However, the experimental conditions used, such as the type and characteristics of the membrane, carrier liquid and flow separation parameters, all of which can influence the outcome, have been only vaguely reported, thus making the methods difficult to reproduce. For example, details of the surface charge of the membrane and how this varies with the pH of the carrier liquid have not been reported in any of these papers even when its importance has been highlighted [25, 26]. In the particular case of a regenerated cellulose membrane, which has been used in all these previous works, the material properties have been reported to be dependent on the manufacturing process and therefore will vary according to the supplier [27]. Moreover, Ma and collaborators in their study on the evaluation of DNA/Chitosan complexes and free chitosan content by AF4, reported a special regenerated cellulose membrane (RC Z-MEM-AQU-631, amphiphilic from Postnova Analytics) which was better designed and suited for the separation of amphiphilic or cationic polymers over the more regular used regenerated cellulose membrane which presumably causes increased analyte adsorption due to interactions with the membrane. In this case, adjustment of carrier liquid composition, (*e.g.* by modifying ionic strength or pH) has been suggested as an approach to minimise the membrane-sample interactions [27].

Given that the cross-flow is the main driver of the separation by AF4, its optimization plays a major role in this technique; mainly its initial flow rate and the gradual decay of this during the elution. Previous studies (see Table 1) have used different cross flow programmes with linear decay or step-wise decay. However, it has been shown that an exponential decay is preferred for broadly distributed samples that contain both polymer and aggregates. In

addition to this, this elution profile offers a more uniform molar mass selectivity as it increases the resolution [28]. This becomes important in the analysis of chitosan which has been shown to not always form true solutions of individual macromolecules as the presence of aggregates frequently occurs [20, 29, 30].

In this study, the parameters discussed above have been considered in the development of the method by AF4 presented here. The method has been tested to characterise MW of different types of chitosan of variable source and degree of acetylation. A comparison of results with the far more extensively conventional technique SEC is provided.

2. Materials and Methods

2.1 Materials

A series of both commercial and research chitosan samples derived from chitin of different biological sources, namely crustacean shells, squid pen and fungi, were analysed. Further specifications of each sample are presented in Table 2. Apart from the Protasan® sample, which was the hydrochloride salt, all were in the neutral form. Their degree of acetylation (%DA) was either determined by ^{1}H NMR in our laboratory or documented by the manufacturer. To prepare the solutions (carrier liquid) for AF4 and SEC studies, glacial acetic acid (Fisher Scientific, Loughborough, UK) and sodium acetate trihydrate (Merck, Dorset, UK) were used. MilliQ water (18.3 MΩ) was used throughout.

2.2 Methods

2.2.1 Preparation of polymer solutions and carrier liquids

All chitosan samples were subjected to the same dissolution protocol in which the required amount of polymer was dissolved in the corresponding carrier liquid used to carry out the measurement in AF4 or SEC. Samples were subject to magnetic stirring for 24 hours at room temperature (T = 20 °C) to ensure the complete dissolution of the polymer before measurement. The MW of some of the samples used in this study had reported values in the manufacturer's specification or through characterisation performed by other laboratories using similar methodologies. These reported values served as a reference for comparison of the method developed here for AF4 (see the last column in Table 2).

All measurements were performed on an AF2000 Multiflow system from Postnova Analytics, which was set to be operated in both AF4 and SEC mode. The system was coupled with an

online 21 angle, multi-angle light scattering detector, MALS (PN3621), a refractive index detector, RI (PN3150) and a dual wavelength UV detector (PN3211) which was set for these experiments at 280 nm and 220 nm. Although the RI detector served as the principle concentration detector, in some cases the UV signal provided further information about the samples. When the equipment was operated in SEC mode a viscometer detector (PN3310) was also coupled to the system in addition to all the detectors already mentioned. The mobile phase was filtered through 0.1 μm membrane filter prior to use.

2.2.2 AF4 Methodology (AF4-MALS-RI)

During the operation in AF4 mode, the system was equipped with an analytical asymmetric AF4 channel (Postnova Z-AF4-CHA-611) using a 350 μm spacer. The temperature of the channel was controlled by a thermostat (PN4020) set at 30°C for all experiments. For method optimization four main parameters were adjusted in a series of preliminary experiments (data not shown) as it is explained in detail below:

Sample preparation and injection volume. All samples of chitosan were prepared at a concentration of 2 mg/mL and for each run a volume of 50 μL was injected into the system. At this concentration and volume, good signal to noise was obtained. All samples were prepared according to the dissolution protocol describe above. Once samples were fully dissolved, they were passed through a 5μm filter to remove any large particles or flocs that might be present which could interfere with the analysis [31].

Membrane. In the case of chitosan, due to its cationic nature, the conditioning of the membrane becomes very important in order to minimise sample-membrane interactions and favour the polymer elution. In the case of regenerated cellulose, it has been reported that these type of membranes are likely to contain some residual negatively charged groups

caused by the synthesis of the membrane material and therefore this is dependent on manufacturer [27]. This could be detrimental for the elution of chitosan as some interactions might be expected. Moreover, the zeta potential of regenerated cellulose fibres has been shown to vary with the pH of the solvent in contact [32, 33]. Similarly to previous studies the membrane used was made of regenerated cellulose with 10 KDa cut-off (Z-AF4-MEM-612-10KD, Postnova Analytics). The membrane used in this work had a positive surface charge below pH 4.2 ([34]and Supporting Information SI.2) and therefore, in order to minimise membrane-polymer interactions a pH adjustment approach of the carrier liquid was implemented as suggested in previous studies and described next [27].

Carrier liquid. The solution used as carrier liquid consisted of 0.18 M acetic acid (HAc)/0.02 M sodium acetate (NaAc), pH=3.7. At this pH, the surface of the membrane should be charged positively (ζ~10 mV) this will favour the polymer elution as repulsion forces will be generated between the cationic polymer and the positively charged membrane ([34]and Supporting Information SI.2).

A second carrier liquid, consisting of a buffer solution of 0.3M HAc/0.2M NaAc of pH=4.5, was also evaluated. This solution has been reported as a good solvent for chitosan in which formation of aggregates is reduced [18, 35] and has been used in the characterization of molecular weight distribution of some chitosans by multi-detector SEC [36]

Flow conditions: A cross-flow programmed with a time delay exponential decay (TDE) was found as optimal to characterise all the samples of chitosan. This has allowed not only the separation of molecular aggregates present in solution from the free polymer but also their quantitative characterization. After injection at 0.20 mL/min, sample was focused for 6 min at a rate of 3.30 mL/min and with crossflow (CF) set at 3 mL/min. At the end of the focusing period and a transition period of 0.2 min, the profile of the crossflow was gradually decreased

over 60 min through a series of consecutive steps. These were as follows: a) For 0.2 min CF was kept constant at 3 mL/min, b) CF was then decreased at an exponent of decay of 0.40 to 0.22 mL/min over 30 min period, c) CF was further decreased to 0.11 mL/min during 5 min at 0.80 exponent decay and d) CF was finally decreased to 0.06 mL/min at 0.80 power decay over 5 min after which e) CF was kept at this flow (0.06 mL/min) for additional 20 min (see Figure 1). During this entire process, including the focus step, the detector flow was maintained at 0.5 mL/min which ensures detector baseline stability.

Data collection and analysis were performed using NovaFFF software version 2.0.9.9. (Postnova Analytics). Measurements were repeated at least three times per sample and a blank sample (the solution used as a liquid carrier for measurement) was also run. For the RI detector, all calculations were performed on the subtracted detector signals (sample minus blank signals). The RI signal was used together with the MALS data for MW calculations using an average refractive index increment (dn/dc) for chitosan of 0.19 mL/g as reported elsewhere [36, 37]

2.2.3 Size Exclusion Chromatography Methodology (SEC-MALS-RI-Viscometer)

In SEC mode, the system was equipped with a set of three analytical columns plus a guard column from the TSKgel® PW series (Tosoh Bioscience) packed with a hydrophilic polymethacrylate matrix covering a wide molecular weight separation range (from around $3x10^3$ Da up to $8x10^6$ Da PEO equivalent). The columns were connected sequentially as follows: guard, G6000PW, G5000PW and G2500PW and temperature controlled at 30 °C.

Samples of 1.0 mg/mL were prepared according to the dissolution protocol stated above and filtered through 0.2 µm nylon syringe filters (Fisher Scientific) prior to measurement. In some particular cases (samples with medium and high molecular weight as estimated by AF4:

Viscosan, Protasan, HDP 1.6 and Fungi) the sample had to be further diluted in the same solution used as eluent to a lower concentration of 0.5 mg/ml. This was done to avoid column overloading issues which can cause problems in peak shape and erroneous results in calculated parameters.

All samples were injected at a volume of 50 μL and a constant flow rate of 1.0 mL/min using the solution with pH=4.5 as eluent. The total elution volume for each measurement was 50 mL.

Elution profiles were recorded and analysed using NovaSEC software, version 1.5.0.8. (Postnova Analytics). In common with the AF4 work, the SEC measurements were repeated at least three times per sample. The RI and MALS signals were used for the MW calculations using an average refractive index increment (dn/dc) for chitosan of 0.19 mL/g as reported elsewhere [36].

3. Results and Discussion

3.1 AF4 elution profiles of chitosan in 0.18M acetic acid : 0.02M sodium acetate (HAc-NaAc, pH=3.7)

The samples run by AF4 using HAc/NaAc (pH=3.7) as carrier liquid showed patterns of elution profiles represented in Figure 2 which show examples of some of the samples analysed. Each of the plots displays the MALS 90˚ light scattering signal (LS) and RI signal. Samples such as Ch 70/5, Ch85/5 and Fungi chitosans (2A, 2B and 2C respectively) showed profiles where two size populations can be clearly distinguished from the LS trace. For samples such as Protasan, HDP 1.6 and Viscosan (2C, 2D and 2E) the elution profiles showed two unresolved size populations (LS trace with a noticeable shoulder at the end of the profile). The second peak in Figure 2A was attributed to the presence of macromolecular

aggregates of chitosan in solution which have been a point of discussion by several studies on chitosan characterisation [20, 29, 30]. This shows the robustness of the AF4 method proposed in this study which allows the clear separation of these aggregates from the non-aggregated polymer, allowing the accurate determination of the polymer molecular weight. Existence of aggregates of chitosan in acidic solution is well-known and has been researched and discussed in several studies [29, 30]. Aggregates are normally a hindrance in molecular weight determination by static light scattering, therefore, separation by AF4, is clearly an advantage. Also, AF4 allows the full characterisation of the aggregates present such as their concentration and size. In the particular case of the chitosans analysed here, the fraction of the aggregate present was determined in all cases to be small and less than 4% of the total mass (see Table 3) as highlighted by the small RI signal in the region of the elution profile where aggregate is detected by light scattering. The combination of both signals (LS and RI) in this technique is a real plus for its use in aiding the full interpretation of results**.** Although the polymer and aggregate peaks are not fully resolved in every sample, molecular weight calculation of the polymer peak alone can be carried out with a reasonable degree of accuracy and precision to yield values that are broadly in agreement with other techniques such as SEC. Of course, the MW of the whole sample (polymer plus aggregates) and the MW and size of the aggregates can also be determined in AF4 (see supplementary information SI.1 and SI.5).

3.2 AF4 elution profiles of chitosan in 0.3M HAc/0.2M NaAc *(pH=4.5) and analysis of aggregates*

The solution with pH=4.5 has been reported as a "good solvent" for chitosan, as it promotes the full solubilisation of the polymer while preventing the formation of aggregates [18, 20].

That is, one that avoids the loose conformation of the polymer by balancing the electrostatic forces between the repeat units of the polymer (mostly repulsive forces between the amino groups present in chitosan) and the solvent. Here, this solution was tested as a carrier liquid to evaluate its effect on the aggregate as analysed by AF4. The results by AF4 showed that aggregates are still present even under such solution conditions (Figure 3B). When sample Ch 70/5 was prepared and analysed by AF4 in the solution with pH=4.5, but filtered using syringe filters of nylon material of 0.2 µm or 0.45 µm (Fisherbrand^{TM}) prior to measurement (as it would be for SEC), the elution profile, obtained showed the complete disappearance of the second peak attributed to the aggregate (Figure 3C and 3D). This result shows that the reported lack of aggregates in the SEC method using the buffer solution of 0.3M HAc/0.2M NaAc of pH=4.5 is due to the filtration rather than the choice of solvent.

It has been suggested that the use of higher cross flow rates (> 0.5 mL/min) [38] can induce the formation of aggregates, specifically at the focusing stage where the higher concentration of the polymer is present [39]. However, the lack of an aggregate peak in the filtered sample suggests that higher cross-flow rates like the ones used in this study (3.0 mL/min) do not lead to the formation of chitosan aggregates. If that were the case, then even after sample filtration the aggregate peak would still show. However, this was not the case as can be seen clearly from the plots (Figure 3C and 3D).

In other works with AF4, the appearance of a second peak after the cross-flow stops, has also been observed [40]. This was suggested to be caused by retained material which finally elutes with the cessation of the cross flow. It should be noted that the aggregate peak we are observing appears at around 30 minutes in the elution when the cross flow is still active (>0.5 mL/min) and so cannot be due to this effect.

Despite the reproducible light scattering traces, it proved to be very difficult to obtain reliable quantitative MW results for the samples run on AF4 using the pH=4.5 solution. This is mainly due to the poor quality RI baselines caused by the high salt content in this solution.

3.3 Comparison of AF4 and SEC for molecular weight determination of chitosan

Results of MW obtained by AF4 were compared to those obtained by SEC. Although attempts were made to perform the two types of analysis using the same carrier solution this was not possible. As explained above, the pH of the HAc/NaAc (pH=3.7) solution favours a better elution by AF4. Similarly, it was found that the buffer solution of 0.3M HAc/0.2M NaAc of pH=4.5, as reported previously, worked better for SEC experiments. The fact that the aggregates are still present when using this solvent does not represent a problem since, as a prerequisite of the SEC technique, the samples always need to be filtered through 0.20 or 0.45 μm filter before analysis. Filtration of the polymer solution as shown by this study, and in agreement with others, does remove the fraction of aggregates present. This might explain why in the work of Brugnerotto et al. [36] these aggregates were not present in their SEC MW distribution curves. In this work, Figure 4 shows the MALS signals by AF4 for samples showing the existence of an aggregate in solution (by the appearance of a second peak) in comparison to those obtained by SEC where no indication of aggregation in the elution profile is observed (single elution peak obtained). A comparison of the results gathered from both techniques under their most satisfactory solvent and separation conditions was performed to assess their correlation. The difference in weight-average molecular weight (M_w) derived from the two techniques, the polymer fraction from AF4 and the SEC result with the aggregates physically filtered out, was not significant. (see Figure 5A). Good linear correlation was found between both techniques with R^2= 0.998 independent of the solution being used (see Figure 5B). Table 3 shows a summary of the MW distributions parameters

obtained by AF4 (using 0.3M HAc/NaAc, pH=3.7) and SEC (using the solution with pH=4.5).

Recoveries of samples from AF4 and SEC were calculated from the peak area obtained from the concentration detector and the known dn/dc and comparing it to the original mass of sample injected [39]. Sample recoveries obtained from AF4 ranged from 38-68%. These recoveries might suggest loss of material caused by sample adsorption due to potential polymer-membrane interactions taking place. Such interactions might be increased by the action of CF as seen from additional experiments carried out (see material SI.1 in the supplementary section). When sample Ch70/5 was run by direct injection, (CF=0) the recovery attained was >95%, comparable to (96%) that was obtained by SEC for the same sample. However when elution of this sample was carried out under the action of CF, the recovery of the sample was reduced to <55% which might be an indication of enhanced interaction with the membrane due to the force of the CF. Interaction might be also influenced by the degree of deacetylation or other parameters related to the intrinsic nature of the polymer which could be subject to further studies. Analysis of the total molar mass in both cases (direct injection and cross flow) did not vary considerably, thus suggesting that loss of material is non molecular weight dependant. Studies of AF4 with other polymers have also shown loss of material due to CF action and also showed that even when recoveries were low, reliable quantitative results could be obtained [41]. In general, AF4 recoveries for cationic polymers have been considered high if these are ~80% [26]. Here, calculated values of M_w were comparable and in good correlation with those obtained by SEC and are also in broad agreement to those reported in suppliers' specifications and to those obtained from measurements performed at other laboratories (see Table 2). This supports the reliability of the method used even with the somewhat low recoveries achieved. Moreover, the method developed here allowed the separation of polymeric chitosan from the molecular aggregates

that are present in solution. Based on this discussion, direct comparisons of recoveries which is technique dependant between AF4 and SEC or even between this study and previous studies is not feasible unless all experimental conditions are closely matched, particularly those that influence the loss of material.

The values of Mark-Houwink constants *K* and *a* at T=25°C in the buffer solution of 0.3M HAc/0.2M NaAc of pH=4.5 for other chitosans with varying DA have been previously reported as *k*= 0.079-0.057 mL $\cdot g^{-1}$ and *a*= 0.79-0.825 argued to be concurrent with the semi-rigid character of chitosan [44]. The values determined in this work by SEC with viscometer, RI and MALS detection under the same solvent conditions but at T=30 °C, for chitosans varying DA and MW, resulted in values of *a*= 0.73-1.0 spanning a wider range than those previously reported by Rinaudo [44]. **The value of *a*=1 found for the low molecular weight (LMW) samples analysed (Ch-70/5 and Ch85/5) may be related to the fact that in this case, the MW of the chain is such that its length lies in the range of the persistence length, as suggested in previous studies for these low MW samples [45], Values of *a*≥1 have also been previously reported for chitosans and attributed to degrees of acetylation below 84%** [46, 47]

4. Conclusions

The method developed for AF4 was successfully applied to calculate MW of a series of chitosans of varying biological source and degree of acetylation and has been shown to be

robust to separate and quantify aggregates present in solution. These aggregates, however, have been shown to be present in a relatively small fraction of the total mass (<4.0%). It has been also demonstrated that **e**ven in solutions reported as good solvents for chitosan (e.g. a buffer solution of 0.3M HAc/0.2M NaAc of pH=4.5) chitosan aggregates persist as detected by AF4. Lower recoveries of chitosan during AF4 experiments compared to SEC are shown to not greatly influence the calculated MW values.

The results of M_w obtained by AF4 showed excellent correlation with those obtained by SEC within the size exclusion range of the columns used ($R^2 = 0.99$). Filtration of the sample through a small pore size filter (≤ 0.45μm) before SEC can effectively remove the presence of aggregates from low MW chitosans without impacting the calculated molar mass. This, however, is not the case for high molecular weight chitosans where filtration may result in loss of sample.

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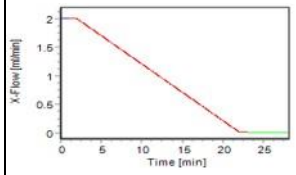
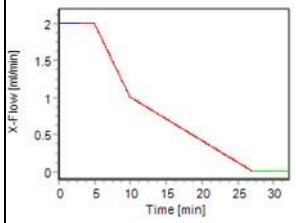
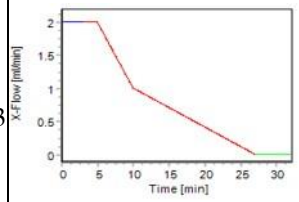
Table 1. Summary of previous studies and experimental conditions used for characterisation MW distribution of chitosan by AF4

Reference	Aim of the study	AF4 System	Membrane	Carrier Liquid	Sample Conc. (mg/mL)	Injected Mass (µg)	dn/dc (mL/g)	CROSS FLOW PROFILE[a]	MALS (Fitting model)
(Jonassen, *et. al.*, 2012)	Evaluation of MW of ultrapure chitosan (Protosan UP CL 213, Novamatrix) used to prepared nanoparticles of Chitosan	AF2000 (Postnova Analytics, Germany) •RI PN3140 (Postnova) •MALS PN3070 with 7 angles 35-145° (Postnova)	**RC** (Z-MEM-AQU-425 N, Postnova) 1000* cutoff	NA	5.0 in 0.01M NaCl	100	NA		Zimm-type
(Augsten and Mäder, 2008)	Characterisation of molar mass distributions of different Chitosans from Primex, Island (FG80- batch TM661; FG85- batch TM611; FG90- batch: TD132; FG95- batch TM1885; FG 95-batchTM1360; FG 95-batchTM1369).	Eclipse F (Wyatt Technology Europe) •Dawn EOS detector with 18 angles(Wyatt) •RI RI-101 (Shodex)	**RC** (Nadir C0010F, Microdyn-Nadir GmbH) 10 KDa cutoff	0.02M acetic acid: 0.08M sodium acetate (pH=4.2) with 0.2g/ L sodium azide	2.0	200	0.181		Zimm-type degree of 1
(Mao, *et. al.*, 2007)	Analysis of Chitosans and trimethyl chitosans	Eclipse F (Wyatt Technology Europe) •Dawn EOS detector (Wyatt) •RI RI-101 (Shodex)	**RC** (Microdyn Nadir) 10 KDa cutoff	0.1M acetic acid/acetate buffer pH=4.2 0.02(w/v) sodium azide	2.0	200	0.181 for pure chitosan 0.145±0.03 for Trimethyl chitosans		Zimm-type

*No units reported

[a]Sketched CF profiles using NovaFFF software version 2.0.9.9 (Postnova Analytics) as interpreted by the description provided in papers

NA= Information not available

Table 2. Detailed description of chitosan samples analysed from varied biological sources

Source	Chitosan sample code	Manufacturer[a]	%DA	Batch number	Previous reported Mw (g/mol) [analytical technique and labpratory]
Crustaceans Shells Waste	**70/5**	HMC+	12[b]	212-170614-01	29,000 [SEC-DRI-MALS, Münster University, Germany] 30,500 [SEC-DRI-MALS, Postnova Analytics, UK]
	85/5	HMC+	15[c]	212-290814-02	Not Available
	Viscosan	FlexiChem	30-60 (37)[c]	NAS-099	Not Available
	Protasan (UP-CL 213)	Novamatrix	10-30[c]	BP-0805-04	150,000-400,000[c] 307,000 [AF4,Jonassen et al., 2012] 177,000 [SEC, Postnova Analytics, UK]
Squid Pen	**HDP1.6**	NBS	1.6[b]	LYO-1106	123,900 [SEC-DRI-MALS, University of Lyon, France]
White Mushroom	**Fungi (740063)**	Sigma	40[c]	STBC5292V	60,000-120,000[c]

[a] HMC+ = Heppe Medical Chitosan (Halle, Germany); FlexiChem (Utran, Sweden); Novamatrix (Oslo, Norway); NBS = University of Münster (Germany), Nanobiosaccharides EU project;; Sigma-Aldrich (Darmstadt, Germany).

[b] As determined by ^{1}H NMR

[c] Value or range as reported by the manufacturer

Table 3. Summary of results (mean average values n=3) of chitosan MW measured by Multi-detection AF4 and SEC and Mark-Houwink constant *a* and *k* in Rinaudo's solvent (pH=4.5) estimated at 35°C

Chitosan Sample	% DA	AF4 (0.18 M HAc)/0.02 M NaAc, pH=3.7)					SEC (Rinaudo's solvent: 0.3M HAc/0.2M NaAc, pH=4.5)						
		$\overline{Mw}$ (g/mol)	$\overline{Mn}$ (g/mol)	$\overline{PD}$ (M / M_n)	Recovery %	Mass Fraction aggregate % (2nd peak elugram)	$\overline{Mw}$ (g/mol)	$\overline{Mn}$ (g/mol)	$\overline{PD}$ (M / M_n)	Recovery %	Intrinsic viscosity $\overline{\eta}$ (dL/g)	Viscometric constants	
												a	k x 10^{-5} (mL/g)
70/5	12	27,460 ± 155	20,533 ± 146	1.34 ± 0.02	52.8 ± 0.3	< 1.0	29,267 ± 289	18,033 ± 404	1.6 ± 0.03	96 ± 0.6	0.72 ± 0.02	1.00	2.29
85/5	15	40,100 ± 56	23,410 ± 573	1.71 ± 0.04	51.0 ± 0.2	< 1.0	38,967 ± 702	22,367± 231	1.7 ± 0.05	98 ± 1.4	0.96 ± 0.01	1.00	2.25
Fungi	40	129,850 ± 3,606	69,885 ± 191	1.86 ± 0.05	57.8 ± 1.6	< 1.0	130,333 ± 8,145	71,633 ± 8,599	1.8 ± 0.11	95 ± 0.3	3.3 ± 0.02	0.83	26.32
HDP1.6	1.6	171,900 ± 566	63,010 ± 2,164	2.73 ± 0.08	67.5 ± 0.5	1.6 ± 0.1	152,333 ± 13,650	76,900 ± 11,873	2.0 ± 0.13	86 ± 8.2	3.4 ± 0.04	0.73	69.50
Protasan	10-30	182,600 ± 1970	106,437 ± 12,507	1.73 ± 0.2	40.3 ± 0.2	3.9 ± 0.3	195,333 ± 3055	129,667 ± 5,774	1.5 ± 0.05	79 ±0.9	5.7 ± 0.2	0.78	49.08
Viscosan	37	274,667 ± 1,950	190,067 ± 1,041	1.45 ± 0.00	37.6 ± 0.2	< 1.0	267,667 ± 7,506	166,667 ± 16,503	1.6 ± 0.12	92 ± 1.4	7.7 ± 0.13	0.81	33.97

Figure 1. International Journal of Biological Macromolecules, Y. Gonzalez-Espinosa, B. Sabagh, E. Moldenhauer, P. Clarke, M. Collado-González and F. M. Goycoolea

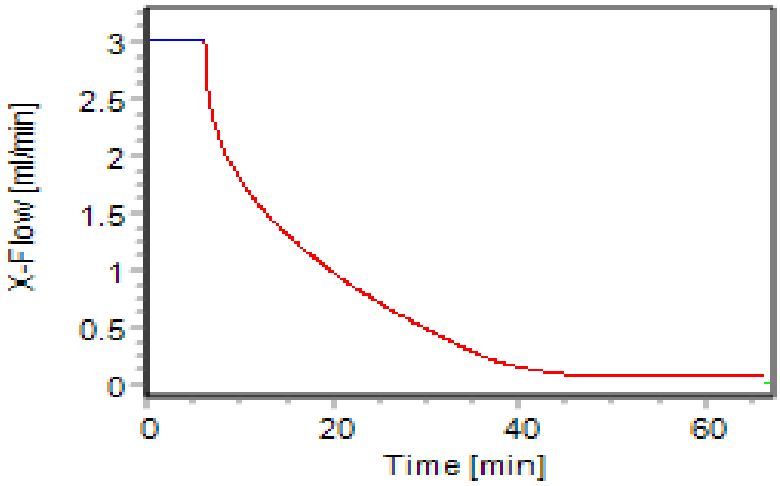


Figure 2. International Journal of Biological Macromolecules, Y. Gonzalez-Espinosa, B. Sabagh, E. Moldenhauer, P. Clarke, M. Collado-González and F. M. Goycoolea

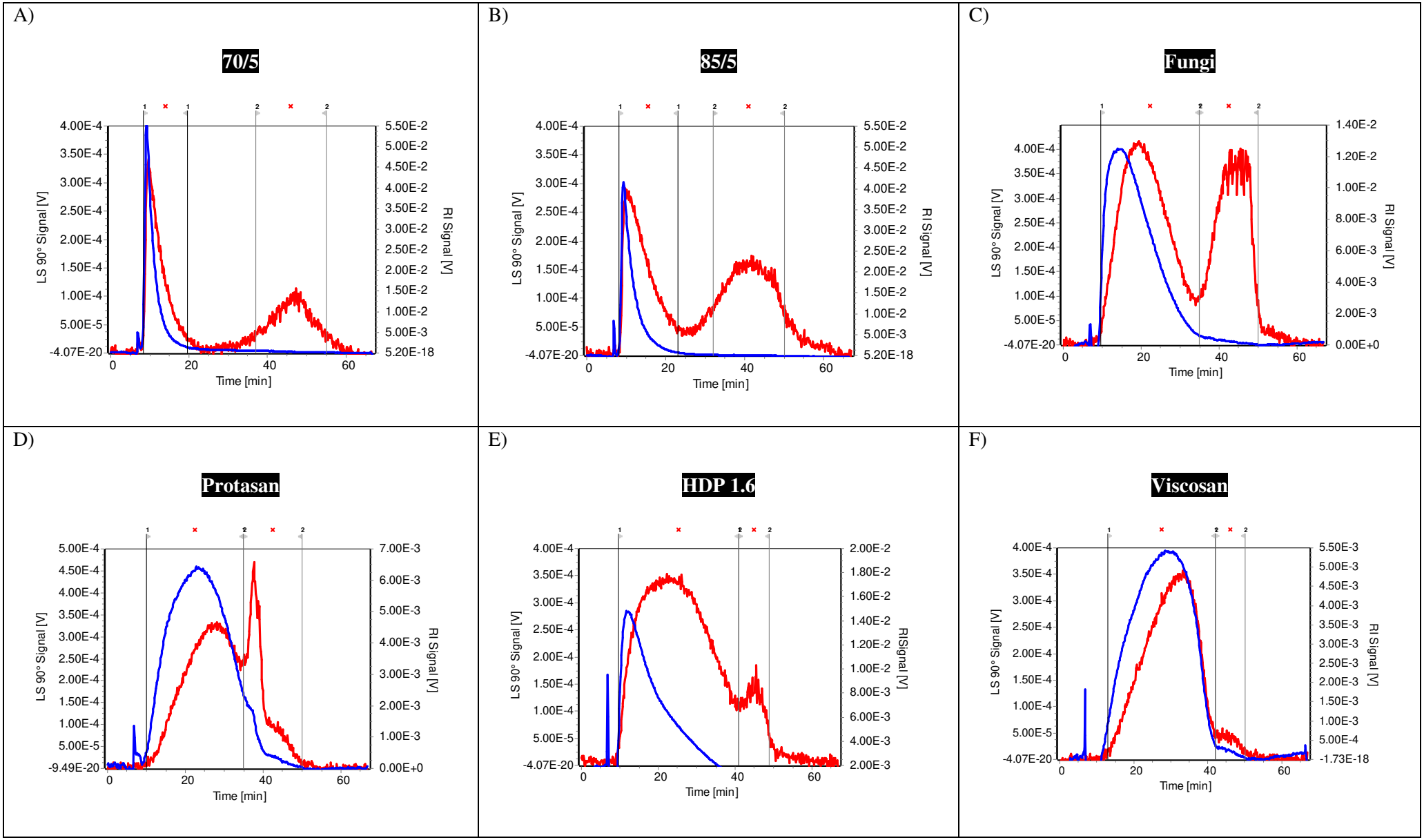


Figure 3. International Journal of Biological Macromolecules, Y. Gonzalez-Espinosa, B. Sabagh, E. Moldenhauer, P. Clarke, M. Collado-González and F. M. Goycoolea

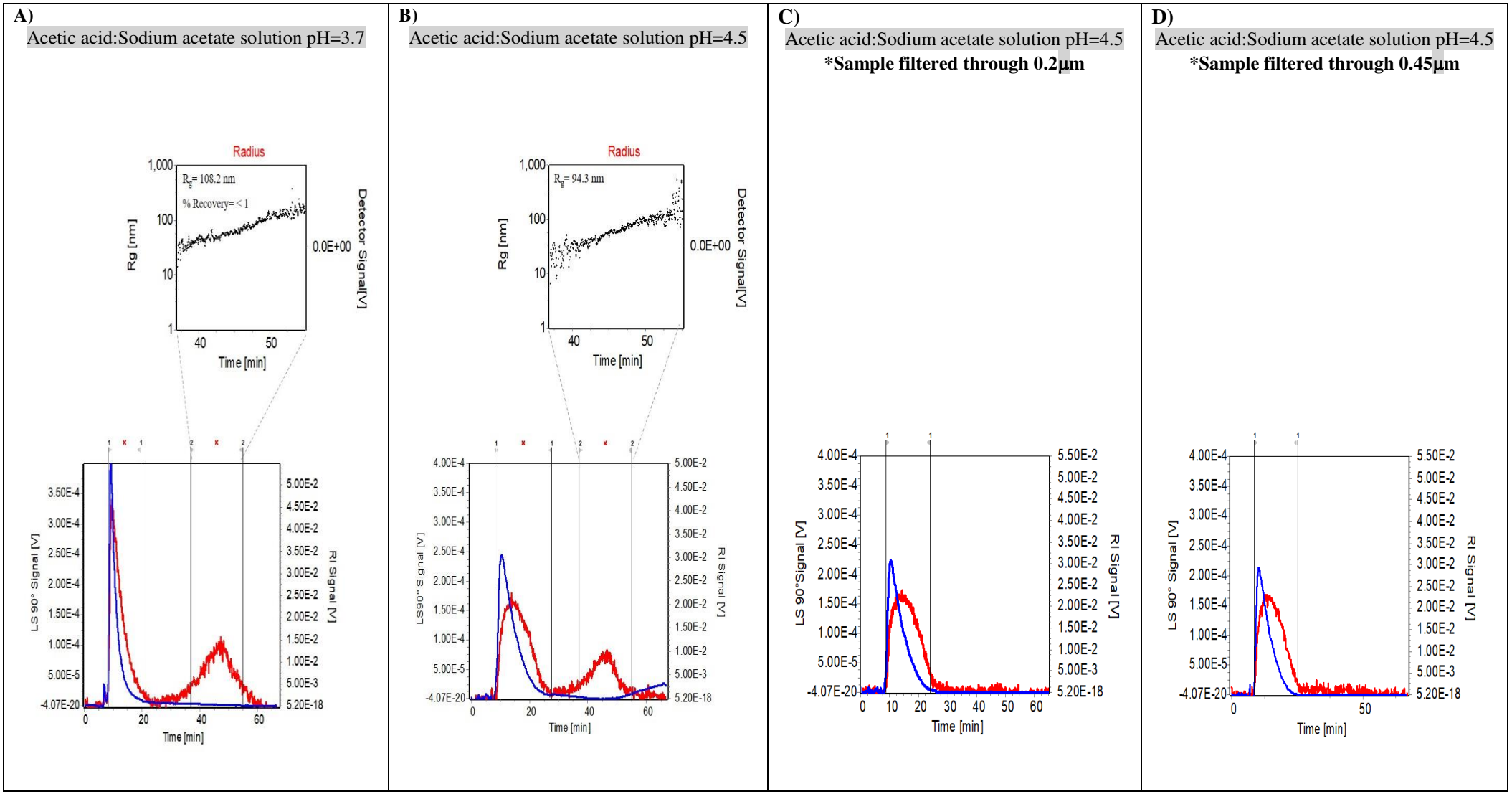


Figure 4. International Journal of Biological Macromolecules, Y. Gonzalez-Espinosa, B. Sabagh, E. Moldenhauer, P. Clarke, M. Collado-González and F. M. Goycoolea

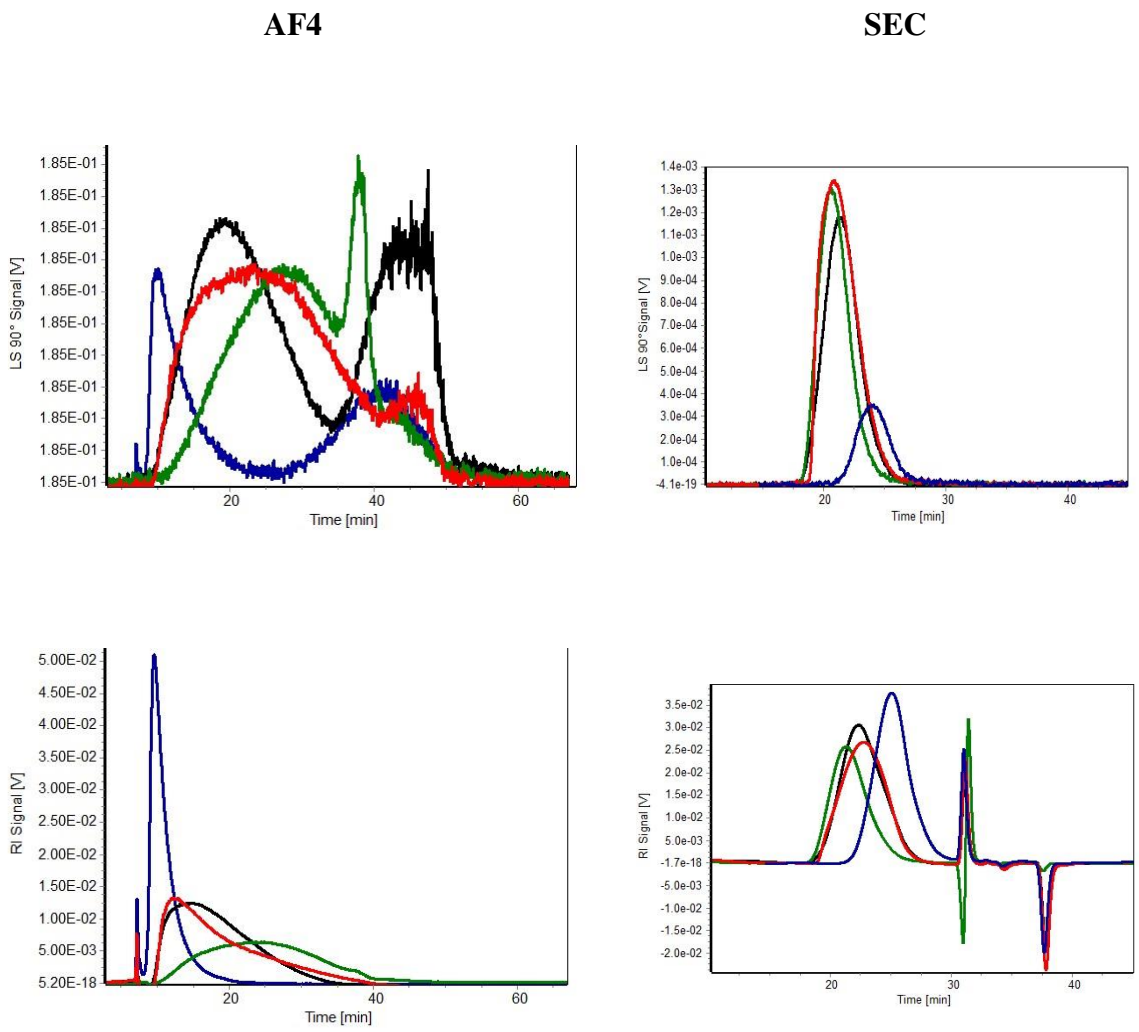
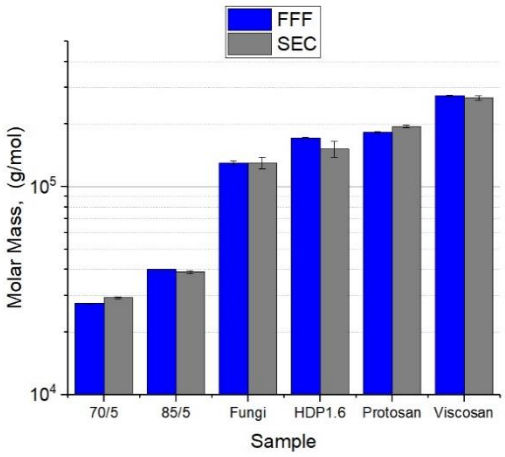
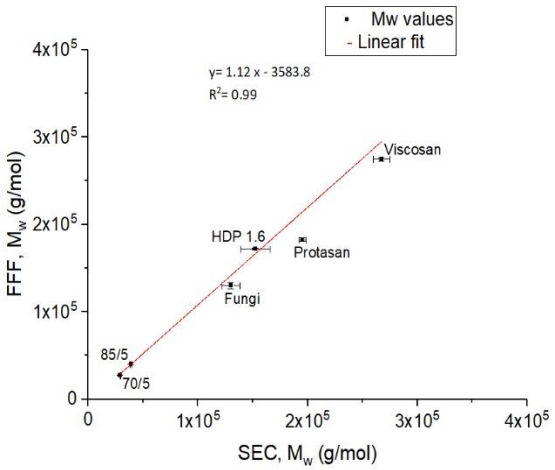


Figure 5. International Journal of Biological Macromolecules, Y. Gonzalez-Espinosa, B. Sabagh, E. Moldenhauer, P. Clarke, M. Collado-González and F. M. Goycoolea.

A.



B.



Captions to Illustrations

Figure 1. Graphical representation of the crossflow (CF) programmed with time delay exponential decay profile. Note that CF along the elution never falls to zero but stays at steady flow rate of 0.06 mL/min.

Figure 2. Representative characteristic patterns of elution of different samples of chitosan by AF4 combining light scattering at 90° (–) and RI signals (–) using as a carrier liquid 0.18 M acetic acid (HAc)/0.02 M sodium acetate (NaAc), pH=3.7. The region in the graphs between vertical lines on the right indicates the presence of a macromolecular aggregate of chitosan present in solution. Void time is at 6.9 min.

Figure 3. AF4 Elugrams of sample Ch 70/5 run on regenerated cellulose membrane 10KDa cut-off and under different solvent conditions: 0.18 M acetic acid 0.18/0.02 sodium acetate solution pH=3.7(A) and 0.3M HAc/0.2M NaAc solution pH=4.5 (B,C and D). Samples in A and B without filtration, while samples in C and D filtered through 0.2µm and 0.45µm, respectively. – LS 90° signal and – RI signal. Rg (black dotted line is presented) above each elugram where a fraction of aggregate was present.

Figure 4. Molecular aggregates of chitosan as detected by light scattering signal (LS 90°) in AF4, second peak in the fractogram (top left plot) in comparison to a single peak obtained for the elution profiles by SEC (top right plot) indicating absence of aggregates. Bottom plots RI signals for AF4 and SEC (left and right plots respectively). In this case indicating the concentration contribution to the second peak obtained is small. Elution profiles correspond to the following samples: — 70/5, — HDP 1.6, — Fungi and — Protasan.

Figure 5. Graph A. Weight Average Molar Mass, M_w of different chitosans as characterised by AF4 (using Acetic acid:Sodium acetate solvent pH=3.7) and SEC (using solvent pH=4.5) and correlation factor between measurements (Graph B.)

SUPPLEMENTARY INFORMATION

Characterisation of chitosan molecular weight distribution by multi-detection asymmetric flow-field flow fractionation (AF4) and SEC

Y. González-Espinosa[*a], B. Sabagh[b], E. Moldenhauer[c], P. Clarke[b], M. Collado-González[a] and F. M. Goycoolea[a]

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[b]*Postnova Analytics UK Ltd., Unit 64, Malvern Hills Science Park, WR14 3SZ, Malvern, Worcestershire, UK*

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SI.1 LOW RECOVERIES IN AF4 NON-MOLECULAR WEIGHT DEPENDENT (COMPARISON OF MOLAR MASS OF DIRECT INJECTION VS CROSS FLOW SEPARATION)

Table SI.1. Comparison of molar Mass and recoveries in AF4: direct injection VS cross flow separation for Sample Ch 70/5 in solvent at pH=3.7

Method	M_w Whole peak (g/mol)	M_wFree polymer (g/mol)	Mass injected (mg)	Mass calculated from RI (mg)	Recovery %
Direct[1]	78270	NA	20	18.931	94.7
Direct[1]	77110	NA	20	19.523	97.6
CF active	69390	29630	100	56.782	56.8
CF active	72870	30330	100	58.307	58.3

[1] Elution of material carried out under no action of cross flow (CF=0)
[2] Elution of material carried out under cross flow action
NA-Not applicable

SI.2 REGENERATED CELLULOSE MEMBRANE SURFACE CHARGE AT DIFFERENT pH VALUES

Experimental conditions

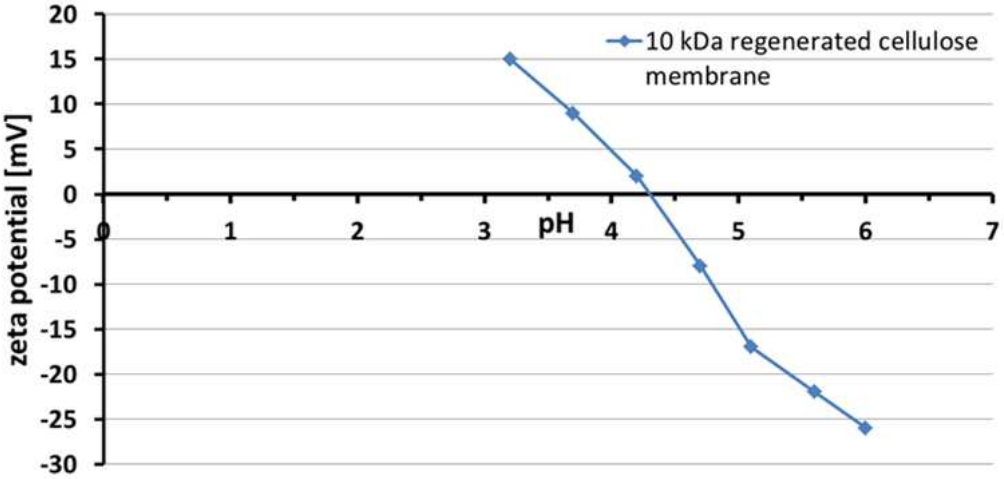
Method:	Streaming current measurement
Device:	SurPASS Electrokinetic Analyzer
Manufacturer:	Anton Paar
Measurement cell:	SurPASS adjustable gap cell
Membrane:	10 kDa regenerated cellulose
Distance between membranes:	100 µm
Electrolyte:	1 mM KCl

All measurements started at the intrinsic pH of a freshly prepared 0.001 mol/l KCl solution, i.e. neutral pH, pH was then adjusted with 0.05M HCl.

Results:

pH	**Zeta potential**
6	-26
5.6	-22
5.1	-17
4.7	-8
4.2	2
3.7	9
3.2	15
4.3	**Isoelectric**

Standard deviation determined from 4 repetitive pressure ramps for data evaluation was < 4%


20
15
10
5
0
-5
-10
-15
-20
-25
-30
zeta potential [mV]
0
1
2
3
pH
4
5
6
7
10 kDa regenerated cellulose membrane

SI.3 DETERMINATION OF THE INCREMENT OF REFRACTIVE INDEX dn/dc FOR CHITOSAN (Ch 70/5) in solvent pH=3.7 USING A REFRACTIVE INDEX DETECTOR form POSTNOVA ANALYTICS PN3150

CALIBRATION:

Sodium Chloride in water dn/dc=0.17035

Solution number	Concentration NaCl (g/100mL)	Experimental Data (mV)
1	0.0526	95.20
2	0.0860	153.50
3	0.1296	230.37
4	0.1679	297.13
5	0.2024	361.00

RI Calibration factor= 1.038816

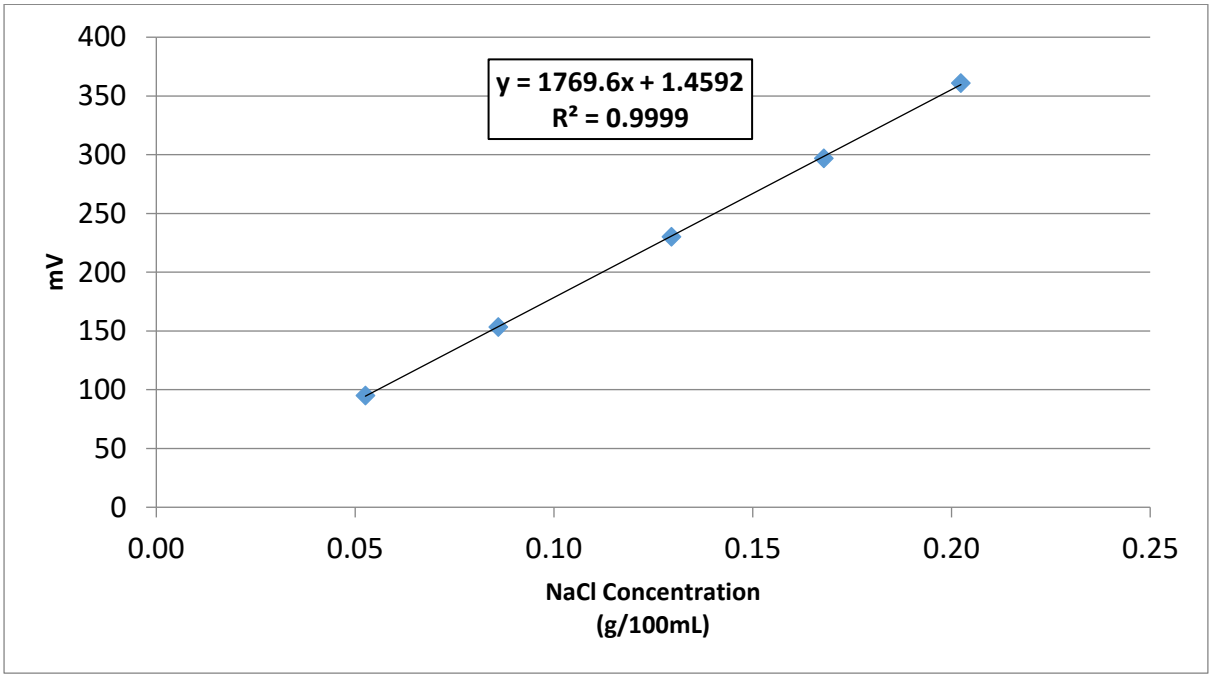


Figure SI.3.1 Calibration for RI detector with NaCl

Determination of refractive index increment of chitosan Ch 70/5 in solvent pH 3.7 using detector PN3150 (Postnova Analytics)

Experimental Data Input		Calibration Constant K	Calculated Data		
(g/100mL)	(mV)		Concentration (g/mL)	Diff. RI n-n0	(n-n0)/c
0.0520	91.6667	1.0388	0.0005	0.0001	0.1831
0.0830	148.7667	1.0388	0.0008	0.0002	0.1862
0.1330	239.1333	1.0388	0.0013	0.0002	0.1868
0.1690	303.8333	1.0388	0.0017	0.0003	0.1868
0.2050	367.1000	1.0388	0.0021	0.0004	0.1860

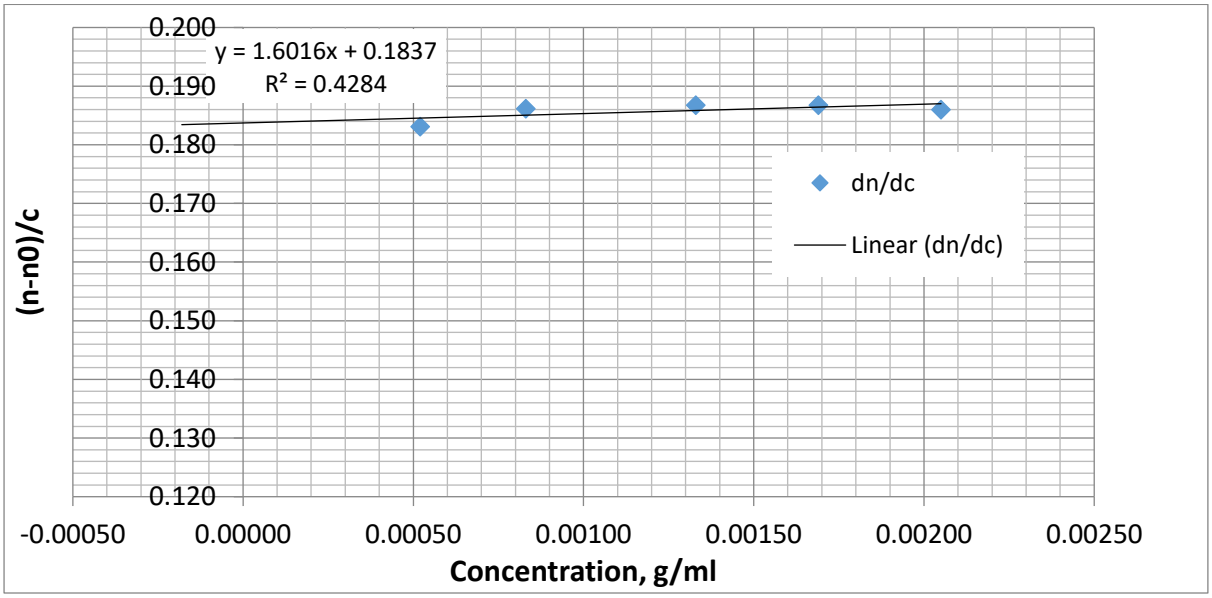


Figure SI.3.2 Refractive Index increment (dn/dc) of Chitosan in acetate buffer pH=3.7 using PN3150 detector

dn/dc= 0.184

SI. 4 DLS measurements of Ch70/5 chitosan solution in two different acetic acid: sodium acetate solutions (pH=3.7 and pH=4.5)

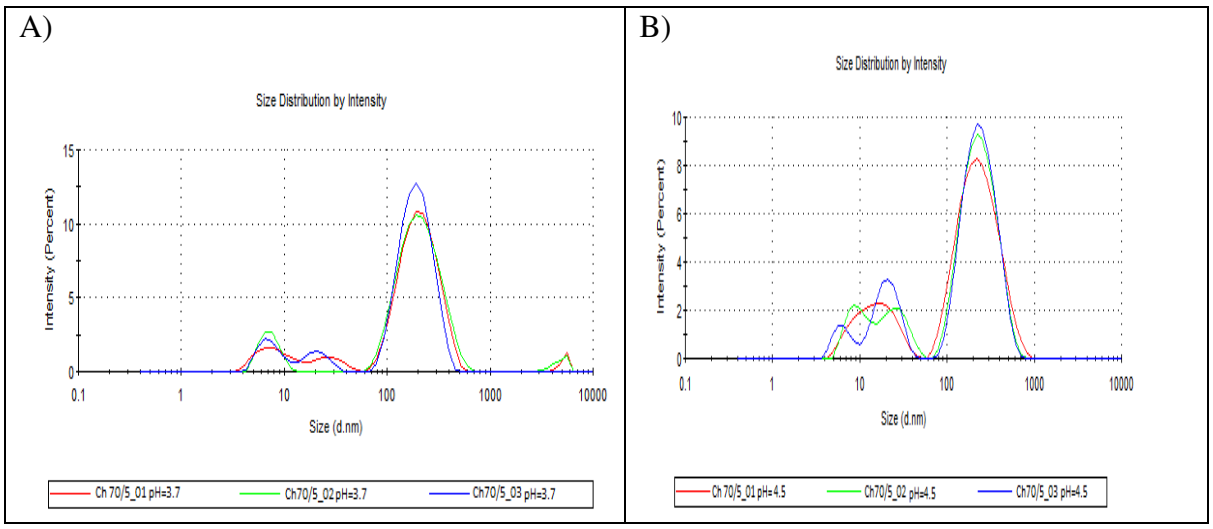


Figure SI.4.3. Particle size by DLS of Ch 70/5 in solution under two differente solvent conditions: A) 0.18M acetic acid : 0.02M sodium acetate pH=3.7 and B) 0.3M acetic acid : 0.2M sodium acetate pH=3.7

SI.5 AF4 ELUGRAMS WITH RADIUS OF GYRATION (Rg) AND MOLAR MASS

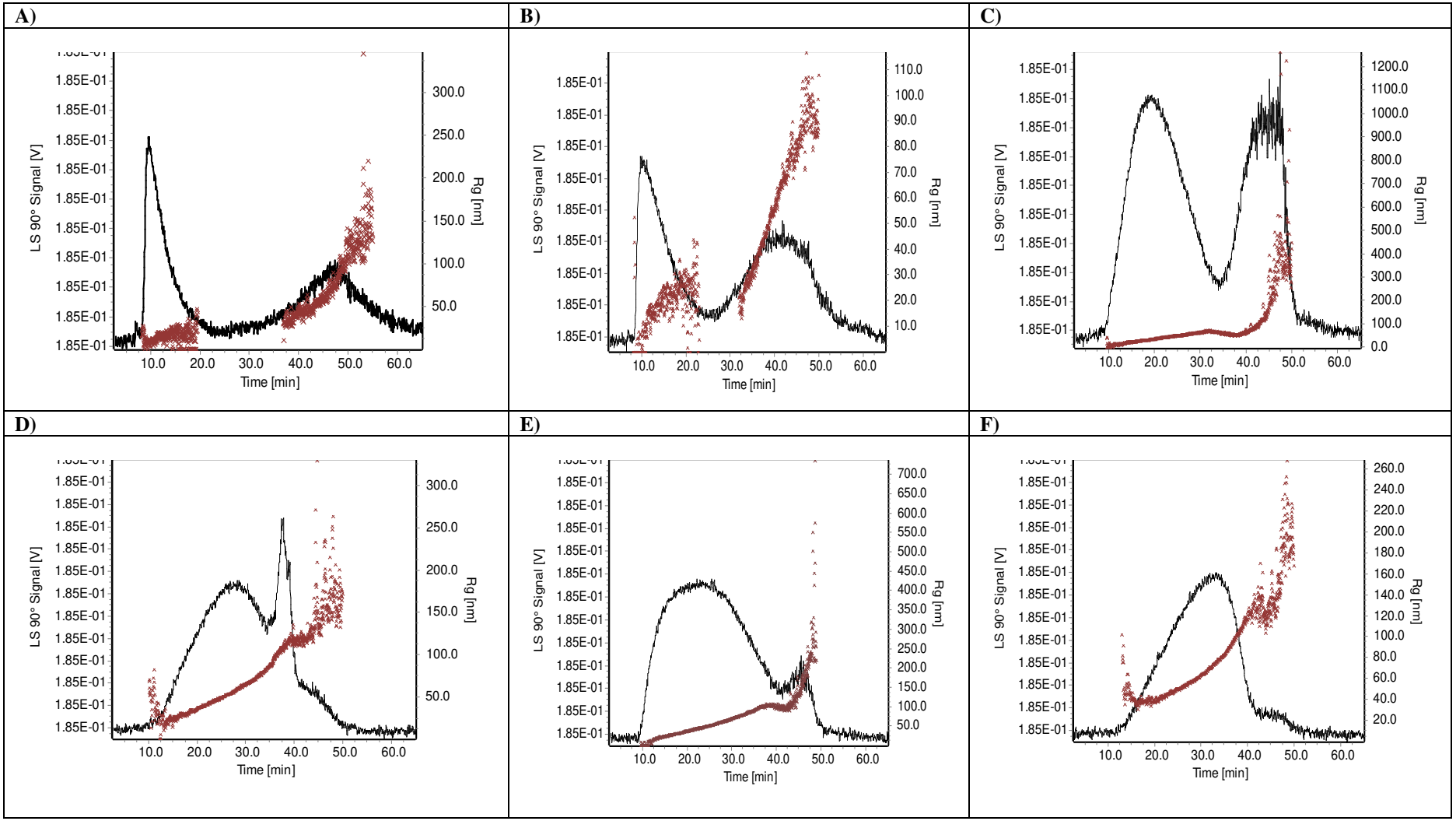


Figure SI.5.1Elugrams (— Light scattering at 90°) showing radius of gyration (*) of different samples of chitosan by AF4: A) Ch 70/5, B) Ch 85/5 C) Fungi, D) Protasan, E) HDP 1.6 and F) Viscosan using as a carrier liquid 0.18 M acetic acid (HAc)/0.02 M sodium acetate (NaAc), pH=3.7.

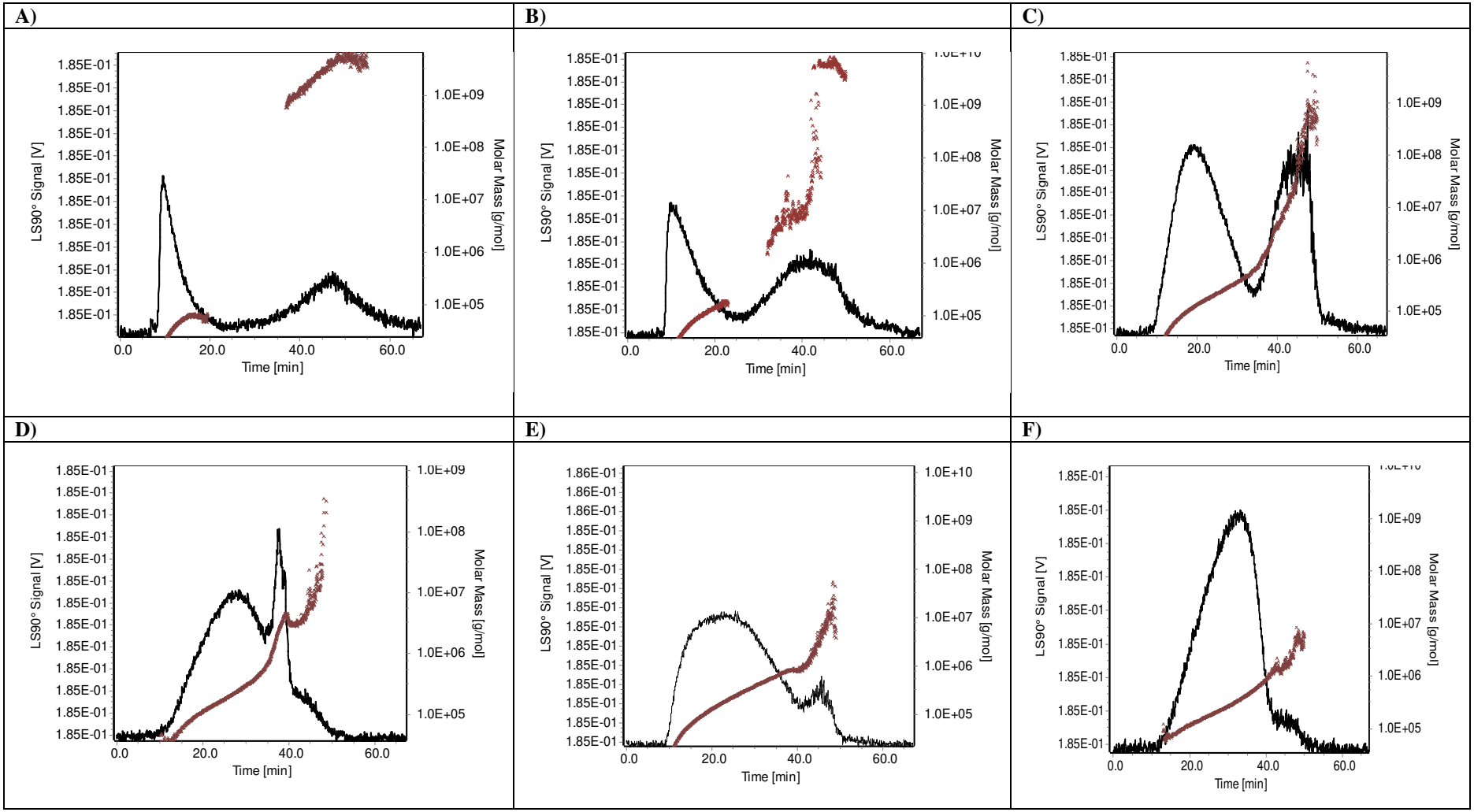


Figure SI.5.2Elugrams (— Light scattering at 90°) showing Molar mass (*) of different samples of chitosan by AF4: A) Ch 70/5, B) Ch 85/5 C) Fungi, D) Protasan, E) HDP 1.6 and F) Viscosan using as a carrier liquid 0.18 M acetic acid (HAc)/0.02 M sodium acetate (NaAc), pH=3.7.