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Oat spelts xylan molecular mass estimation by size exclusion chromatography

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Abstract

This work addresses the investigation of the parameters that may affect the molecular mass estimation of oat spelts xylan (OSX) by size exclusion chromatography (SEC). These parameters, relative to sample preparation and SEC operating conditions, are: xylan concentration, the solvent characteristics, the eluent pH and ionic strength. The SEC experiments were performed on a Sephacryl S-400 HR column. Glycine–NaOH buffer with the pH ranging from 9 to 12.5 was used as eluent. The OSX was dissolved in aqueous solutions of NaOH with concentrations ranging from 0.2 to 0.5 M. The results showed that the xylan concentration and the eluent ionic strength have no significant effect on the determination of the molecular mass. The NaOH concentration in the OSX solution and the eluent pH, have a great influence on the determination of the molecular masses. This is explained by possible changes of OSX–water interactions leading to different hydrodynamic volumes, upon different solution characteristics.

Keywords: Xylan; Molecular mass; Conformation; Size exclusion chromatography

1. Introduction

Xylan is a polypentose hemicellulose occurring in plant cell walls and displaying a wide range of compositions, molecular sizes and structures depending upon in its source (Ebringerova, 1992; Puls, 1997). It consists of a main chain of D-xylopyranose (xylose) units linked by β-glucosidic bonds (Simionescu & Rozmarin, 1972). Besides xylose, there are other saccharide residues, bonded at the two free OH groups of carbons C2 and C3 of the xylopyranose residue (Puls, 1997; Simionescu & Rozmarin, 1972). These saccharide residues can be bonded directly to the main chain or to the one side residue (Verbruggen, Beldman, & Voragen, 1998). Therefore, there are a great variety of xylans with different: degrees of polymerisation of the poly-D-xylopyranose main chain, degrees of substitution, side residues, and the side chain length. This complex and wide variety of structures has a direct effect on the xylan properties particularly solubility. In the literature, there is a distinction between water-soluble and water-insoluble xylans (Ebringerova, Hromadkova, Alfodi, & Berth, 1992; Ebringerova, Hromadkova, Burchard, Dolega, & Vorwerg, 1994). Some authors divide the water-insoluble fraction according to their solubility in different solvents, especially in strong alkaline solutions (Verbruggen, Beldman, & Voragen, 1995).

The determination of the molecular mass is highly dependent on all these factors. For instance, by high performance size exclusion chromatography (HPSEC), oat spelts xylan (OSX) was reported to have weight-average molecular masses about 20 kDa (Eremeeva & Bykova, 1993). However, for rye arabinoxylans, the reported values are 519–770 kDa, and for wheat 255 kDa (Girhammar & Nair, 1992). Although these values refer to xylans of different sources, this spread of molecular masses for the same type of polymer is quite surprising. Moreover the molecular masses reported for cereal arabinoxylans depend not only on the source of extraction, but also on the method used for their estimation (Izydorczyk & Biliaderis, 1995) and the sample preparation procedure. For water-soluble wheat arabinoxylans, molecular mass values obtained by conventional sedimentation equilibrium ultra-centrifugation were about 60–65 kDa (Andrewartha, Philips, & Stone, 1979; Girhammar & Nair, 1992). This is much lower than the value of 850 kDa (Gruppen,

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Hammer, & Voragen, 1992) obtained by light scattering for alkali-extractable wheat arabinoxylans. Even for the same method of determination, very different values are reported. For water extractable wheat arabinoxylans reported molecular masses determined by size exclusion chromatography (SEC) include: 800–5000 (Fincher & Stone, 1986), 70–1000 (Fincher & Stone, 1974) and 217 kDa (Girhammar & Nair, 1992).

The great differences of the molecular masses can be explained by different polymer conformations or by chain aggregation (Dhami, Harding, Elizabeth, & Ebringerova, 1995), which may arise from different sample solubilisation methods or operating conditions. Recently, Dervilly-Pinel, Thibault, and Saulnier (2000) showed that the chains of water-soluble arabinoxylans from wheat flour are semi-flexible. This feature allows these polysaccharides to adopt a random coil conformation in solution. Therefore, the hydrodynamic volume of the macromolecule depends on the solvent characteristics.

As discussed above, the literature data are very controversial and a systematic study of the parameters influencing the determination of the molecular masses is required. In this work, the OSX molecular mass is estimated by SEC. After the characterisation of the polymer in terms of the neutral sugar residues content, this paper investigates the influence of the OSX concentration, the NaOH concentration used in OSX solubilisation, the eluent pH and the eluent ionic strength on the estimation of OSX molecular mass by SEC. The aim of this work is not the absolute determination of OSX mass, but how the polymer solubilisation conditions and the eluent characteristics influence the molecular mass estimation.

2. Experimental

2.1. Analytical methods

The raw material for this study was OSX, produced by Sigma, USA. Two types of sugar analyses were performed for polymer characterisation:

1. The phenol–sulphuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) was used to determine the total neutral sugar content.
2. The OSX was characterised in terms of the saccharides residues by GC analysis of alditol acetates (Albershheim, Nevis, English, & Karr, 1967), using inositol as the internal standard. The alditol acetates were separated and quantified on a Shimadzu gas chromatograph GC-14B with a J&W Scientific DB-225 capillary column (30 m × 0.25 mm). The column temperature was set at 200 °C for 11 min, then raised to 220 °C at 20°/min and after 4 min raised to 235 °C at 10°/min for 5 min. Nitrogen was used as carrier gas.

2.2. Xylan solubilisation

Prior to SEC experiments, OSX solubility tests were performed, in order to solubilise xylan at 1 g/l in aqueous NaOH with the concentration ranging from 0.05 to 0.5 M. The suspension was stirred and heated, if necessary, until a clear solution was obtained.

2.3. Size exclusion chromatography

The runs were performed on a 16 × 660 mm Sephacryl S-400 HR (Pharmacia, Sweden) column. The eluent was fed with a peristaltic pump P-1 (Pharmacia, Sweden) at 1 ml/min flow rate. The sample injection volume was 200 μl. A refractive index detector, Gilson model 133, was used and the chromatograms recorded on paper using a Philips, PM 8252 recorder.

The chromatographic media used is stable up to pH 11 and for short periods up to pH 13. The eluents were prepared with glycine–NaOH 0.1 M buffer with pH ranging from 9 to 12.5 and containing 0.1 M NaCl. In order to investigate the effect of the eluent ionic strength, eluents without NaCl and 1 M NaCl were prepared. The eluent and the OSX solutions were filtered through 0.45 μm membranes (Gelman Laboratory) prior to use.

The column was calibrated with dextrans T10, T40, T70, T110, T500 (Pharmacia Fine Chemicals products) and dextran 2 000 000 (Sigma). The exclusion limit of the column was determined with blue dextran (Pharmacia, Sweden) and corresponds to a molecular mass of ca. 2000 kDa. The calibration data was fitted by an equation of the type:

$$\log M = a + bV_e \quad (1)$$

where M is the molecular mass and V_e is the elution volume. The values of a and b were slightly different for the six eluents used: pH 12.5 0.1 M NaCl, $a = 8.40$, $b = -0.036$; pH 12.5 without NaCl, $a = 8.47$, $b = -0.038$; pH 11.8 0.1 M NaCl, $a = 8.31$, $b = -0.034$; pH 10.8 0.1 M NaCl, $a = 8.33$, $b = -0.035$; pH 9 0.1 M NaCl and 1 M NaCl, $a = 8.56$, $b = -0.039$.

Even taking into account the different stiffness and the mass per unit length of OSX and dextrans we considered that this calibration allows a good estimation of the molecular masses and the comparison between the values determined at different conditions. In any event there is a lack of more appropriate polysachharide standards.

2.4. Chromatogram processing

The chromatograms recorded were processed numerically. The area under the curve of the refractive index signal versus elution volume corresponding to the peak was divided into n partial areas. Every partial area ($Area_i$) was approximated to the corresponding trapezoid area. To each incremental elution volume corresponds a mean molecular

mass, M_i, calculated from the calibration equation. The number average molecular mass M_n, and mean weight average molecular mass M_w were calculated as follows:

$$M_n = \frac{\sum \text{Area}_i}{\sum \text{Area}_i/M_i} \quad (2)$$

$$M_w = \frac{\sum \text{Area}_i M_i}{\sum \text{Area}_i} \quad (3)$$

Another parameter, mean molecular mass, M_m, was determined as the mass corresponding to peak maximum. Polydispersity is defined by the ratio M_w/M_n.

The cumulative molecular mass distribution, $F(M_i)$, was calculated as the sum of the partial areas and normalised through the division by the total peak area:

$$F(M_i) = \frac{\sum_{i=0}^{i} \text{Area}_i}{\sum_{i=0}^{n} \text{Area}_i} \quad (4)$$

where n is the total number of partial areas. The molecular mass distribution $f(M_i)$ was obtained by numerical differentiation of the normalised cumulative distribution.

2.5. Ionic strength

The ionic strength of the eluents was calculated assuming total dissociation of NaOH and NaCl. The concentration of the dissociated glycine was calculated from the dissociation constant, pK = 9.60 (White, Handler, & Smith, 1973). The H_3O^+ and OH^- concentrations were determined from the eluent pH value.

3. Results and discussion

Analysis by gas chromatography of the alditol acetates was carried out to quantify the neutral saccharide residues, yielding the composition shown in Table 1. Although OSX is mainly composed of xylan there is a significative amount of other sugars particularly arabinose and glucose (Table 1).

The preliminary tests revealed that OSX is soluble in solutions of NaOH and the polymer dissolution is favoured by high concentrations of NaOH in the range from 0.2 to 0.5 M.

Table 1
Neutral sugar residues of OSX

Arabinose (%)	Xylose (%)	Mannose (%)	Galactose (%)	Glucose (%)
9.8	81	<0.2	1.4	7.6

A typical chromatogram obtained for OSX solubilised in a 0.5 M NaOH solution is presented in Fig. 1, showing the presence of two peaks. A minor peak is observed at the exclusion limit of the column (ca. 59 ml), and a major one around 100 ml. After collecting separately each of the peaks, the analysis for the total sugar content revealed that the second peak contains about 95% of the total sugar of the OSX. Consequently, only the second peak was taken into account for OSX characterisation. The first peak can be related to some products with high molecular masses or chain aggregation. Chromatograms, like the one shown in Fig. 1, were recorded with long time intervals, proving a good reproducibility with time in terms of elution volume and surface area.

The numerical processing of the chromatograms allowed the determination of molecular mass distribution and its respective cumulative distribution function, as displayed in Fig. 2. The molecular mass distribution is well fitted by a Gaussian distribution function.

3.1. Effect of xylan concentration

In order to investigate the influence of the OSX concentration in the molecular mass estimation by SEC, three solutions containing 0.4, 1.0 and 2.0 g/l of OSX in aqueous 0.5 M NaOH were loaded in the column and eluted with a pH 12.5 buffer. The corresponding chromatograms are presented in Fig. 3. There are also two peaks, and the ratio of their areas is approximately constant for the three OSX concentrations.

The results obtained from numerical processing of these chromatograms, M_m, M_w and M_n, are shown in Table 2. It is

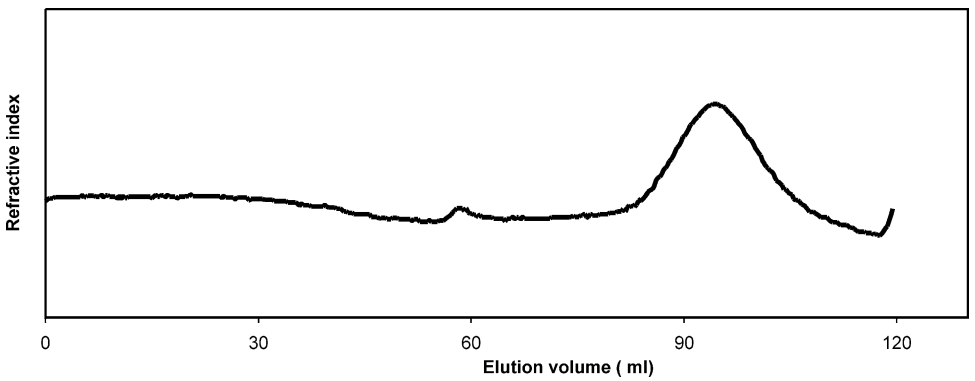

Fig. 1. Chromatogram of 1 g/l OSX solution in aqueous NaOH 0.5 M and eluting at pH 9.

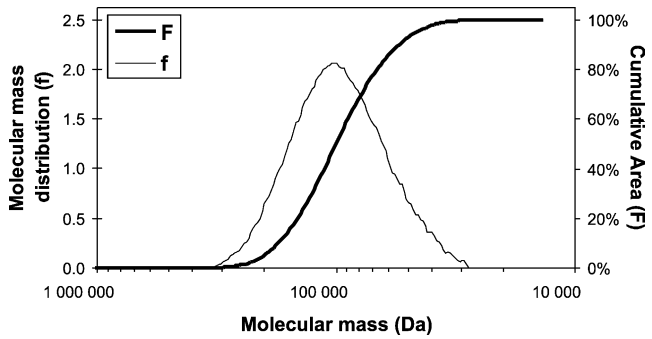

Fig. 2. Molecular mass distribution (f) and cumulative mass distribution (F) of OSX solution at 1 g/l in aqueous 0.5 M NaOH eluted at pH 9.

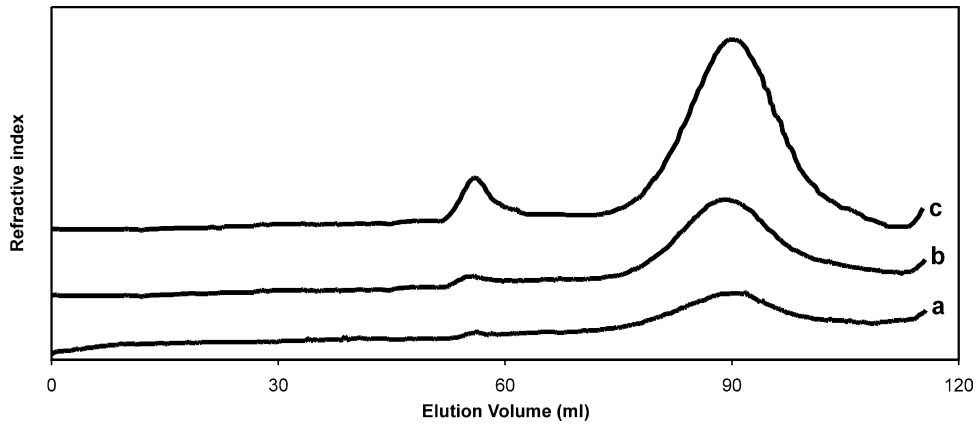

Fig. 3. Comparison of three chromatograms corresponding to three different OSX concentrations: a—0.4; b—1; and c—2 g/l. The solutions were prepared in NaOH 0.5 M and the eluent pH was 12.5.

observed that there is no significant influence of the xylan concentration on the determined molecular masses. Based on these results, all the subsequent experiments were carried out with 1 g/l xylan solutions.

The polydispersity values are always under 1.40. This is a rather small value for a natural polymer and could be explained by the fact that the OSX under study was probably an alkaline extract and thus a fraction of the whole natural polymer.

3.2. Effect of NaOH concentration of OSX solution

OSX was solubilised in NaOH solutions ranging from 0.2 to 0.5 M. The chromatograms obtained are compared in Fig. 4. There is a peak shift towards lower elution volumes, i.e. higher molecular masses, with increasing of NaOH concentration, for the two different values of eluent pH tested. The chromatogram corresponding to 0.2 M NaOH solution presents a non-symmetric shape, probably due the presence of the negative water peak at the column total volume.

The M_w values obtained for the different NaOH concentrations are represented in Fig. 5. The average values corresponding to these determinations are shown in Table 3. As discussed above, there is an increase in the apparent polymer molecular mass with the NaOH concentration used for OSX solubilisation. Taking into account the mechanism of SEC, there is no increase of the molecular mass but an increase in the hydrodynamic volume of the macromolecule in the solutions with higher NaOH concentration.

Macromolecules in electrolyte solutions develop electrical double-layers and give rise to electrostatic interactions (Mayers, 1991). The literature concerning the determination of molecular mass by SEC sometimes ignores this effect. In the present work the high electrolyte concentrations and ionic strengths of OSX

Table 2
Influence of OSX concentration on the molecular masses and polydispersity. Xylan in aqueous NaOH 0.5 M eluted at pH 12.5

Xylan concentration	0.4 g/l	1 g/l	2 g/l	Average	Standard deviation
M_m (kDa)	152.4	153.6	148.1	151.4	2.9
M_w (kDa)	170.0	173.6	163.2	168.9	5.3
M_n (kDa)	127.2	129.6	130.7	129.1	1.8
Polydispersity	1.34	1.34	1.25	1.31	0.05

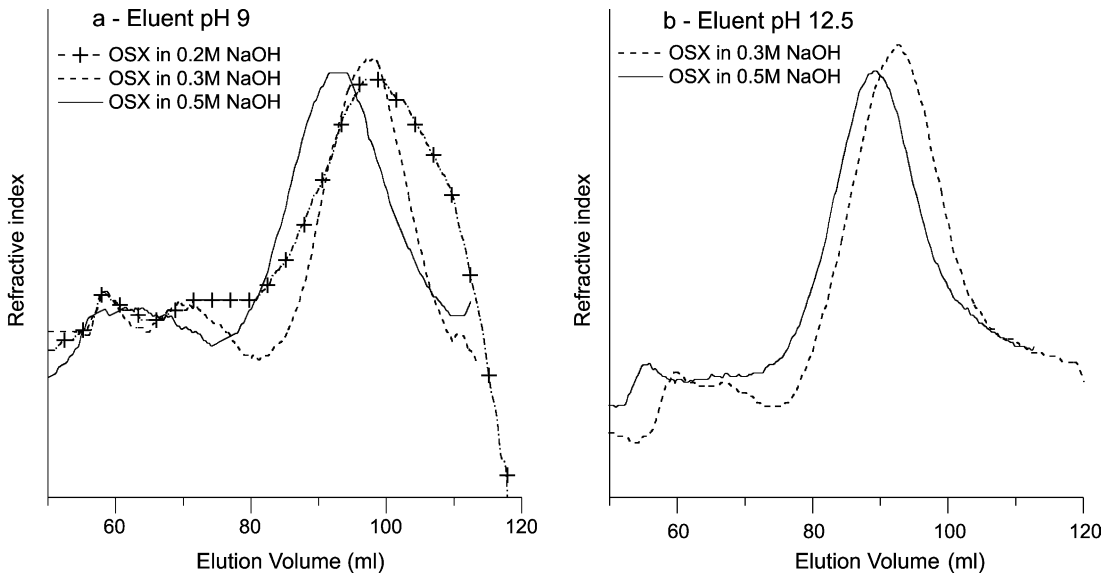

Fig. 4. Comparison of chromatograms corresponding to different NaOH concentration in OSX solution, at two values of the eluent pH.

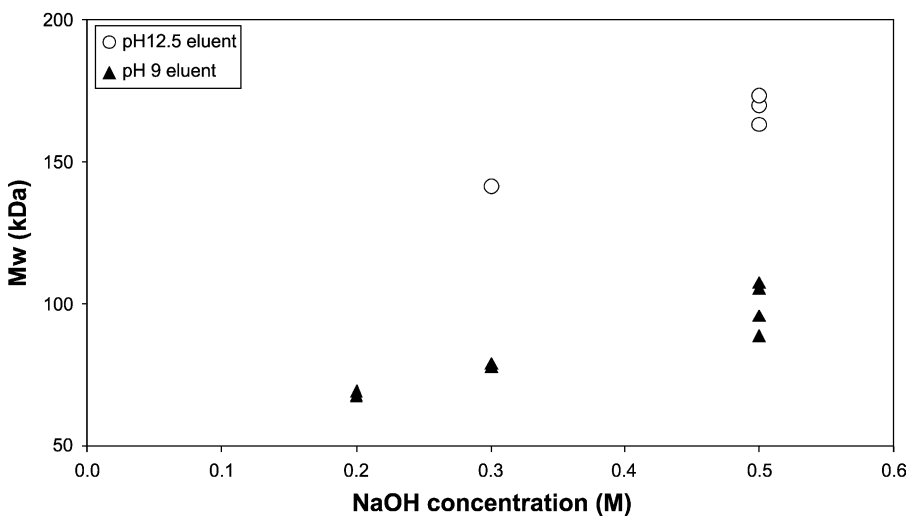

Fig. 5. The influence of NaOH concentration in OSX solutions on the weight molecular mass, M_w, at two values of the eluent pH.

solutions assure the minimization of the thickness of the electrical double-layer and thus electrostatic interactions will be negligible.

The results do not support the hypothesis of chain aggregation, as this phenomenon will preferentially occur in the less concentrated NaOH, where the dissolution of OSX is more difficult. Moreover, if chain aggregation occurs, new peaks should be observed, corresponding to a multiple of the original OSX molecular mass.

The increase of the NaOH concentration leading to higher molecular masses can be attributed to the higher hydrodynamic volumes of the macromolecule/water aggregates as a result of the polymer water sorption (Yarwood, 1999). These sorption mechanisms are very complex (Trommsdorff & Tomka, 1995) and beyond the scope of this paper.

Table 3
The influence of NaOH concentration of the OSX solution on the molecular mass determined at two different pH values of the eluent (9 and 12.5)

NaOH conc. (M)	pH 9			pH 12.5	
	0.2	0.3	0.5	0.3	0.5
M_n (kDa)	58.6 ± 7.3	72.0	89.7 ± 13.3	127.2	151.4 ± 7.2
M_w (kDa)	68.1 ± 2.5	78.5	99.5 ± 14.0	141.1	168.9 ± 13.1
M_n (kDa)	50.5 ± 4.9	64.1	72.2 ± 13.4	112.0	129.1 ± 4.5
Polydispersity	1.36 ± 0.3	1.23	1.29 ± 0.10	1.26	1.31 ± 0.13

Note: the errors were calculated only when three or more determinations of the same sample were done.

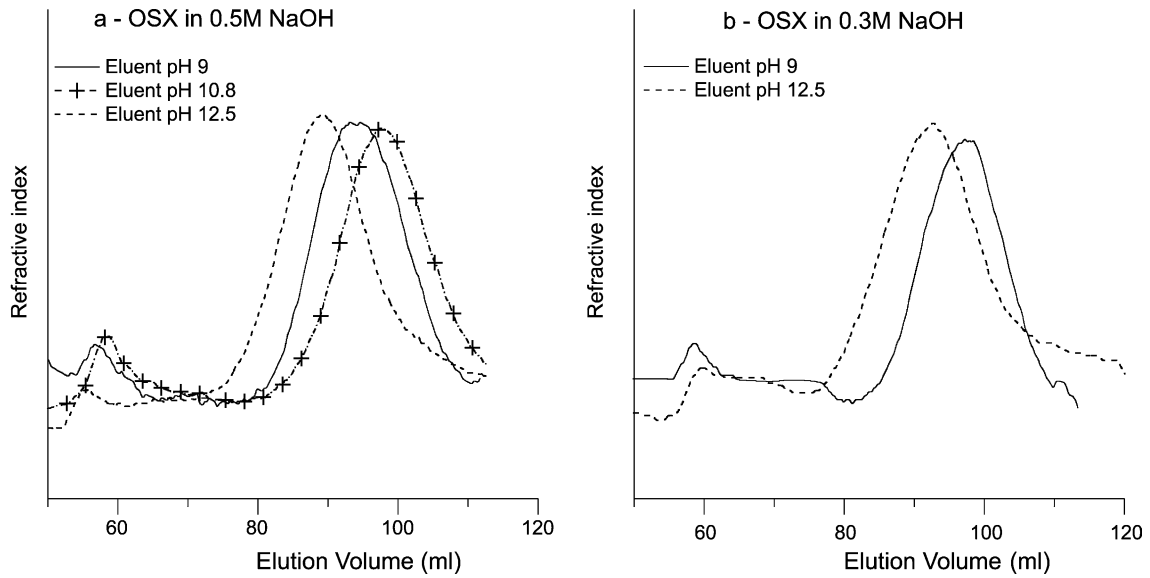

Fig. 6. Comparison of chromatograms corresponding to different values of the eluent pH for OSX solutions with two different NaOH concentrations.

Table 4
Influence of the eluent pH on the molecular mass for two OSX solutions with different NaOH concentrations

	0.5 M NaOH				0.3 M NaOH	
	pH 9	pH 10.8	pH 11.8	pH 12.5	pH 9	pH 12.5
M_m (kDa)	89.7 ± 13.3	68.4	82.5	151.4 ± 7.2	72.0	127.2
M_w (kDa)	99.5 ± 14.0	77.5	89.8	168.9 ± 13.0	78.5	141.1
M_n (kDa)	77.2 ± 13.4	57.3	73.9	129.1 ± 4.5	64.1	112.0
Polydispersity	1.29 ± 0.10	1.36	1.21	1.31 ± 0.13	1.23	1.26

Note: the errors were calculated only when three or more determinations of the same sample were done.

3.3. Effect of eluent pH

The SEC runs of OSX solutions were performed at different values of eluent pH. These ranged from 9 to 12.5 and the chromatograms are displayed in Fig. 6. For the highest value of eluent pH equal to 12.5, there is a pronounced increase in molecular mass for both samples with different NaOH concentrations. In fact, Table 4 reports that the average values of M_m, M_w and M_n, at pH 12.5 are nearly double of those obtained at

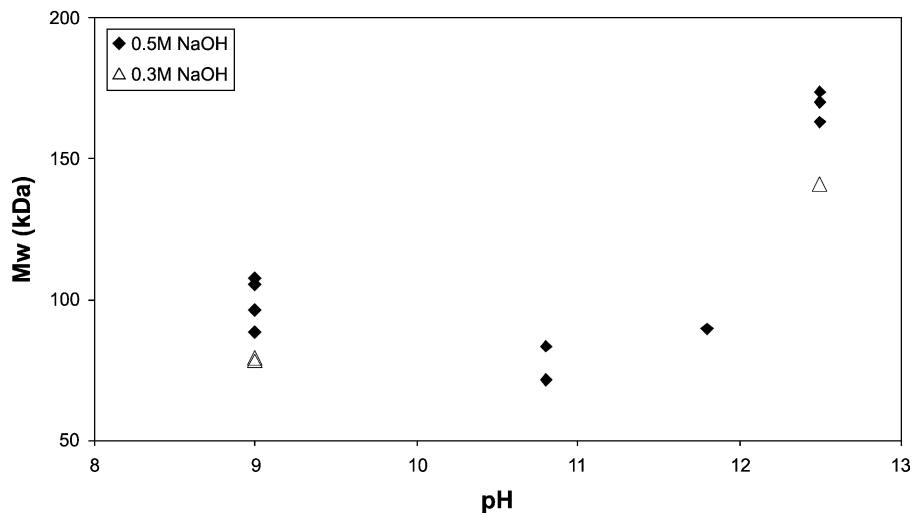

Fig. 7. The influence of eluent pH on the mean weight molecular mass, M_w, for two OSX solutions with different NaOH concentration.

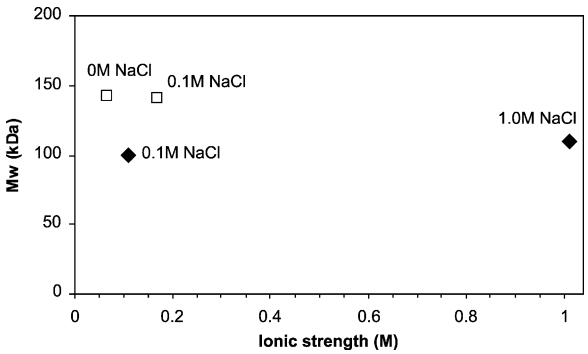

Fig. 8. Influence of the eluent ionic strength on the mean weight molecular masses: OSX solutions in NaOH 0.5 M, eluted with pH 9 buffers containing 0.1 and 1.0 M NaCl (◆); OSX solutions in NaOH 0.3 M, eluted with pH 12.5 buffers containing 0 and 0.1 M NaCl (□) The NaCl concentration is written near the points on the graph.

eluent pH equal to 9. For the intermediate pH values one can observe, in Fig. 6a, that at pH 10.8 there is a small shift towards lower molecular masses. The variation of the mean weight molecular mass (M_w) with eluent pH, presented in Fig. 7, clearly shows that the M_w is almost constant in the pH range 9–11.8 and sharply increases at pH 12.5.

The increase of molecular mass with the eluent pH could be related not only with the pH but also with the increase of the eluent ionic strength. However, the values of eluent ionic strength display a slight increase from 0.12 to 0.17 M in the eluent pH range 9–12.5, which cannot explain the sharp modification of the molecular mass at the highest pH. Further evidence on this negligible effect of the ionic strength on the OSX molecular mass determination was given through the variation of eluent NaCl concentration, as shown in Fig. 8. It was not possible to investigate lower ionic strength due to experimental limitations.

The increase in the polymer molecular masses, or hydrodynamic volume, in higher pH eluent, is probably related to a change in the polymer–water interactions as proposed above to explain the influence of NaOH concentration.

4. Conclusions

The SEC measured OSX molecular masses are strongly influenced by the experimental conditions, namely the NaOH concentration of the OSX solution and the eluent pH. The OSX molecular mass values show an increase with the NaOH concentration of the OSX solution and the eluent pH, while the OSX and the NaCl concentrations have no significant effect on the determinations. This apparent increase of molecular mass is associated to different polymer–water interactions, causing an increase of the macromolecule–water aggregate hydrodynamic volume. Taking into account the results obtained, the spread of molecular masses of OSX obtained by SEC, existing in the literature, can be partially explained by the differences in the experimental conditions used, and that have influence on the hydrodynamic volume of the macromolecule.

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