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Challenges in analysis of high-molar mass dextrans: Comparison of HPSEC, AsFlFFF and DOSY NMR spectroscopy

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ABSTRACT

Dilute solutions of various dextran standards, a high-molar mass (HMM) commercial dextran from *Leuconostoc* spp., and HMM dextrans isolated from *Weissella confusa* and *Leuconostoc citreum* were analyzed with high-performance size-exclusion chromatography (HPSEC), asymmetric flow field-flow fractionation (AsFlFFF), and diffusion-ordered NMR spectroscopy (DOSY). HPSEC analyses were performed in aqueous and dimethyl sulfoxide (DMSO) solutions, while only aqueous solutions were utilized in AsFlFFF and DOSY. The study showed that all methods were applicable to dextran analysis, but differences between the aqueous and DMSO-based solutions were obtained for HMM samples. These differences were attributed to the presence of aggregates in aqueous solution that were less prevalent in DMSO. The study showed that DOSY provides an estimate of the size of HMM dextrans, though calibration standards may be required for each experimental set-up. To our knowledge, this is the first study utilizing these three methods in analyzing HMM dextrans.

1. Introduction

Dextrans produced by lactic acid bacteria (LAB) can improve the texture and viscosity of fermented food products. Since LAB have a GRAS (Generally Recognized as Safe) status, it is preferable to produce the dextrans in situ during fermentation which is a current approach for replacing hydrocolloid additives in food products. As Welman (2009) maintains, this is the most practical and cost-effective way, and also suits the present consumer demand for products with a "natural image". In sourdough, production of dextrans in situ was shown to increase the bread volume, improve the texture, and retard staling of sourdough wheat bread (Katina et al., 2009; Korakli, Rossman, Gänzle, & Vogel, 2001; Tieking, Korakli, Ehrmann, Gänzle, & Vogel, 2003). According to Lacaze et al., dextrans should have a low degree of branching and a high molar mass (HMM) for a positive impact on sourdough bread quality (Lacaze, Wick, & Cappelle, 2007). Determining the structure and macromolecular properties of HMM dextrans is however challenging due to their architectural complexity.

Structurally, dextrans are homopolysaccharides composed of α-glucosyl units. Based on the linkages in the main chain, dextrans are currently divided into three classes: Class 1 dextrans have α-(1 → 6) linkages in the main chain, with α-(1 → 2), α-(1 → 3), or α-(1 → 4) branch linkages; Class 2 dextrans (alternans) have alternating α-(1 → 3) and α-(1 → 6) linkages in the main chain with α-(1 → 3) linked branches, and Class 3 dextrans (mutans) contain α-(1 → 3) linkages in the main chain with α-(1 → 6) side chains (Naessens, Cerdobbel, Soataert, & Vandamme, 2005; Robyt, 1986). Though several studies have addressed the structural properties of dextrans, the topology of the branches in dextrans, which can be single unit and/or elongated with a varying number of glucosyl residues, is not fully understood (Maina, Virkki, Pynnönen, Maaheimo, & Tenkanen, 2011). Studies show that dextrans are complex branched polysaccharides with a ramified structure rather than a comb-like structure. The branches have been estimated to have a molar mass of 29 000 g/mol (~179 glucosyl units) (Ioan, Aberle, & Burchard, 2001).

Similarly, the macromolecular properties of dextrans have been determined in various studies. The methods used include light scattering (Nordmeier, 1993; Setford, 1999; Wu, 1993), analytical ultracentrifugation (Setford, 1999), high-performance size-exclusion chromatography (HPSEC), and asymmetric flow field-flow fractionation (AsFlFFF) (Ioan, Aberle, & Burchard, 2000; Irague et al., 2012; Wittgren & Wahlund, 1997). Most comprehensive studies utilizing light scattering, HPSEC, and AsFlFFF

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have addressed the solution properties of commercial dextrans derived from *Leuconostoc mesenteroides* strains (Ioan et al., 2000; Nordmeier, 1993; Setford, 1999; Wittgren & Wahlund, 1997). Like other complex branched polysaccharides with irregular branching patterns (Gilbert, 2011), native dextrans have a distribution in the density and length of branch chains in addition to a molar mass distribution (Gidley et al., 2010; Vilaplana & Gilbert, 2010). Consequently the size of dextran molecules is not directly related to their molar mass (Gilbert, 2011) which should be considered during data interpretation. Although HPSEC is still the most commonly used technique for separation of dextrans, alternative methods, such as hydrodynamic chromatography (HDC) and column-free AsFlFFF, which are considered gentler separation techniques than HPSEC, are currently being used for HMM polysaccharides.

In addition to the above methods, another potential technique is diffusion-ordered NMR spectroscopy (DOSY), which has been proposed as a versatile tool for estimating the molar mass and hydrodynamic volume (R_h) of uncharged polysaccharides (Viel, Capitani, Mannina, & Segre, 2003). As Nilsson (2009) points out, the term DOSY refers to a method for processing and displaying pulse field gradient (PFG) diffusion NMR data that results in a 2D plot with chemical shifts in one dimension and self-diffusion coefficients D ($m^2\ s^{-1}$) in the other. Antalek (2002) has reviewed how to obtain reliable results and the other methods used to process PFG diffusion NMR data, and a thorough evaluation of the experiment is provided in Johnson's review (Johnson Jr., 1999). By calibrating the diffusion dimension with structurally similar standards, DOSY can be used to determine the molar mass of polymers (Barre et al., 2009; Chen, Wu, & Johnson, 1995; Politi, Groves, Chávez, Cañada, & Jiménez-Barbero, 2006). DOSY experiments are carried out on conventional NMR spectroscopy equipment and therefore the technique is a suitable alternative where HPSEC and AsFlFFF equipment is not available. DOSY is advantageous as it is a non-destructive method for simultaneous analysis of the structure, molar mass and R_h of polysaccharides (Antalek, 2002). Furthermore, DOSY permits the analysis polysaccharide mixtures since, as long as the components have different D values, the NMR spectra in the chemical shift dimension provide the necessary information to distinguish each polysaccharide. This is currently a challenge in HPSEC or AsFlFFF with the existing with detection methods, often entailing additional purification procedures to obtain pure solutions of each polysaccharide.

In this study, HMM dextrans were analyzed with HPSEC, AsFlFFF and DOSY. HPSEC analysis was accomplished in aqueous and DMSO solutions to examine the possible effect of the solvent on the solution properties of the dextrans. AsFlFFF and DOSY were performed in aqueous solutions only. The study began with evaluation of data obtained with the three methods, for commercially available dextran standards with a weight average molecular weight (M_w) ranging from 12 000 to 11 900 000 g/mol. Subsequently, a HMM commercial dextran from *Leuconostoc* spp., and two structurally different HMM dextrans produced by *Weissella confusa* and *Leuconostoc citreum*, which have a potential application in sourdough baking, were analyzed. To our knowledge, this is the first comparative study where these three methods are employed in the analysis of HMM dextrans.

2. Experimental

2.1. Materials

Dextran standards with nominal M_w of 12 000–270 000 and 670 000 g/mol (Dx 12, Dx 50, Dx 150, Dx 270, Dx 670) were purchased from Fluka (Buchs, Switzerland) and standards with nominal M_w of 490 000, 3 500 000, and 11 900 000 g/mol (Dx 490, Dx 3500, Dx 11900) from Polymer Standards Service (Mainz, Germany). Commercial dextran produced by *Leuconostoc* spp. was from Sigma–Aldrich (Steinheim, Germany). The HMM dextrans produced by *Weissella confusa* VTT E-90392 (= DSM 20194, NCDO 1975) and *Leuconostoc citreum* VTT E-93497 were obtained as described previously (Maina, Tenkanen, Maaheimo, Juvonen, & Virkki, 2008). Briefly, the bacteria were grown on MRS agar containing sucrose, at 30 °C for 5 days in anaerobic conditions. The cell mass was harvested and suspended in phosphate buffer saline. Cells were separated by centrifugation and dextran recovered from the supernatant by ethanol precipitation and subsequently lyophilized. Water was purified with a Milli-Q-Plus system (Millipore Corp., Billerica, MA, USA). $NaNO_3$, LiBr, and D_2O (99.9%) were from Merck (Darmstadt, Germany). HPLC-grade DMSO was from Lab-Scan (Dublin, Ireland).

2.2. HPSEC

HPSEC analyses were accomplished using aqueous (0.1 M $NaNO_3$) and DMSO-based (DMSO + 0.01 M LiBr) eluents. The concentration of the samples in aqueous solutions was 2 mg/ml and 3 mg/ml in DMSO and the dissolution time was 4 days. The HPSEC equipment consisted of an integrated autosampler and pump module (GPCmax, Viscotek Corp., Houston, TX, USA), a combined light scattering and viscometric detector (270 Dual Detector, Viscotek Corp.), and a refractive index (RI) detector (VE 3580, Viscotek Corp.). The light scattering detector (λ_0 = 670 nm) included two scattering angles: 7° and 90°. Two OHpak SB-806 M HQ columns (8 mm × 300 mm, exclusion limit 2×10^7, Showa Denko, Ogimachi, Japan) with an OHpak SB-6 (4.6 mm × 10 mm) guard column were used for the water-based eluent. In the DMSO-based system, two LF-804 columns (8 mm × 300 mm, exclusion limit 2×10^6, Showa Denko, Tokyo, Japan) with a LF-G (4.6 mm × 10 mm) guard column were used. The flow rate in both HPSEC systems was 1 ml/min and injection volume 100 μl. The HPSEC data (molar mass, [η], and R_η = viscometric radius), were processed with the OmniSEC 4.5 software (Viscotek Corp.). The dn/dc value of 0.1435 ml/g for dextrans in the water-based eluent (Vink & Dahlstrom, 1967) and 0.072 ml/g for the DMSO-based eluent (Basedow, Ebert, & Ruland, 1978) were used.

2.3. AsFlFFF

AsFlFFF experiments were carried out in aqueous solvent (0.1 M $NaNO_3$) using an AF2000 MT instrument (including software, Postnova Analytics, Landsberg/Lech, Germany) equipped with multi-angle light scattering (MALS, Brookhaven Instruments Corporation, Holtsville, NY, USA) and refractive index (PN 3150, Postnova Analytics) detectors. The MALS detector contains a 30 mW laser as the light source operating at λ_0 = 660 nm with seven scattering angles (35°, 50°, 75°, 90°, 105°, 130°, 145°). Separation occurred in a rectangular channel consisting of a bottom plate with a ceramic frit, a spacer with a thickness of 350 μm, and a top plate with flow outputs. A membrane (regenerated cellulose, cut-off value 10 000 g/mol) was placed on top of the ceramic frit. The pump system included two isocratic pumps and a piston pump for maintaining cross-flow (Pitkänen, Tenkanen, & Tuomainen, 2011). Different cross-flow gradients were used for the standards and the isolated dextran samples. For the standards and the *Leuconostoc* spp. dextran, the exponential decay of cross-flow (exponent 0.2) was used for the separation step starting with a cross-flow of 2 ml/min, and the detector flow was kept constant at 0.5 ml/min during analysis. Because molecules flow in the channel near the membrane, the excess solvent from the upper part of the channel was pumped to the waste at a flow rate of 0.5 ml/min before the detector outlet to intensify the detector signals. For the isolated

Table 1
Weight-average molar mass values ($M_w \times 10^{-3}$ g/mol) and dispersity indices (M_w/M_n) for dextran samples.

Sample	Manufacturer's data[b]			DMSO + 0.01 M LiBr		H_2O + 0.1 M $NaNO_3$			
	Relative calibration		Light scattering	HPSEC		HPSEC		AsFlFFF	
	M_w	M_w/M_n	M_w	M_w	M_w/M_n	M_w	M_w/M_n	M_w	M_w/M_n
Dx 12[a]	11.6	1.43	11.7	8.5	1.1	11.0	1.01	nd	
Dx 50	48.6	1.36	51.0	42.0	1.05	48.0	1.01	50.6	1.27
Dx 150	148	1.47	143	131	1.07	148	1.02	124	1.3
Dx 270	273	1.66	262	223	1.06	267	1.07	232	1.52
Dx 490	490	3.20	1520	379	1.19	1499	2.99	1824	6.19
Dx 670	668	2.01	676	440	1.03	648	1.26	540	2.43
Dx 3500	3500	2.50	3360	1425	1.09	3136	1.06	3102	2.1
Dx 11 900	11 900	4.68	16 700	3866	1.13	10 720	1.13	nd	

[a] Dx 12 = dextran 12 000 g/mol.
[b] Analysis carried out in aqueous solvent.

dextran samples with HMM, a faster decay of the cross-flow was used (exponent 0.1), starting with a lower cross-flow of 1.5 ml/min. The detector flow was 1 ml/min and the injection volume was 50 μl for the standards and 20 μl for the samples. The RI and MALS detectors were calibrated according to instructions from Postnova Analytics (2009) using the bovine serum albumin and polystyrene sodium sulfonate standards. The concentration for the dextran standards and the *Leuconostoc* spp. dextran was 2 mg/ml and the concentration for *W. confusa* and *L. citreum* dextrans was 1 mg/ml (dissolution time 4 days). For data analysis, blank runs were subtracted from the sample and the Berry equation was used for the molar mass and size calculations. In addition to molar mass, the MALS detector enabled R_g to be determined.

2.4. DOSY

DOSY measurements were carried out to determine the self-diffusion coefficients D ($m^2\,s^{-1}$) of the dextran standards and samples. The experiments were performed in D_2O on a Varian Unity INOVA 600 spectrometer equipped with a 5 mm triple-resonance gradient probe-head incorporating three-axis gradient coils, capable of delivering gradient amplitudes up to 31, 31 and 64 G/cm on the *x*-, *y*- and *z*-axis, respectively. Only the gradient along the *z*-axis was varied during the DOSY measurements. DOSY measurements were carried out at 300 K using dilute solutions (<1 mg/ml) of the dextran standards and samples. The standards and samples were exchanged twice with D_2O before analysis. Sample heights in the NMR tube were kept below 4 cm, and the experiments carried out using the bipolar gradient pulse stimulated echo pulse sequences with convection compensation (BPPSTE-CC) (Jerschow & Müller, 1997) to suppress the effects of gradient non-linearity and especially the effect of convection. In all experiments, the gradient amplitude was varied from 1% to 98% of the maximum in 30 steps with a total of 16 transients collected at each gradient amplitude. The amplitudes were calculated according to Eq. (1).

$$G_i = G_{min} + (G_{max} - G_{min}) \times \left(\frac{i-1}{n-1}\right)^2 \quad (1)$$

where i is the gradient index, n is the total number of amplitude steps (31), and G_{max} and G_{min} are the maximum and minimum gradient amplitude values. The diffusion time was kept constant (0.6 s) for all samples and standards while the diffusion gradient pulse duration was optimized for each sample to ensure that at the highest gradient, the signals decayed to at least 5% of the original intensity. Typical values for the diffusion gradient pulse durations were between 1 and 6.5 ms. A 200 μs eddy current recovery delay was applied after each gradient. Polydispersity of the samples analyzed results in multi-exponential decays in the NMR data, which thus require the use of inverse Laplace transformation (ILT) to obtain representative DOSY spectra (Delsuc & Malliavin, 1998; Tramesel, Catherinot, & Delsuc, 2007). In this study, ILT incorporated in NMRnotebook software (NMRtec, France) was utilized.

The self-diffusion coefficients D ($m^2\,s^{-1}$) of the dextran standards were related to M_w according to Eq. (2) (Viel et al., 2003), where K and α are scaling parameters that depend on the molecular architecture of the polymer, the viscosity of the solvent, and the temperature (Barre et al., 2009). The scaling parameters K and α were obtained by calibration with the dextran standards (nominal M_w ranging from 12 000 to 11 900 000 g/mol).

$$D = KM_w^{\alpha} \quad (2)$$

D is also related to R_h by the Stokes–Einstein equation (3), where k ($J\,K^{-1}$) is the Boltzmann constant, T is the temperature (K), and η (P) is the solvent viscosity.

$$D = \frac{kT}{6\pi\eta R_h} \quad (3)$$

3. Results

3.1. Recovery of the samples

Low molar mass (LMM) dextran standards (≤670 000 g/mol) and commercial dextran from *Leuconostoc* spp. were soluble in the solvents utilized and their analysis recoveries were above 80% in both HPSEC and AsFlFFF. The recovery values for HMM standards (≥3 500 000 g/mol) (and also *W. confusa* and *L. citreum* dextranswere lower (<50%). In both solvents the solutions of HMM dextrans were cloudy, indicating the presence of insoluble material. The solutions were therefore filtered before analysis and thus only represent the soluble fraction of these dextrans.

3.2. HPSEC and AsFlFFF analysis of dextran standards

The molar mass data (M_w, M_w/M_n) from the HPSEC and AsFlFFF analyses for the standards are shown in Table 1 together with the values reported by the manufacturer. Except for Dx 490 the results for standards in aqueous solutions were consistent in both HPSEC and ASFlFFF. Furthermore, the values obtained here correspond well with manufacturer's values. The M_w value for Dx 490 from the HPSEC and AsFlFFF analysis in aqueous solvent corresponded only with the value obtained with light scattering by the manufacturer (Table 1). In DMSO, the M_w values for dextrans standards below Dx 270 were relative similar to the values obtained with aqueous solutions (Table 1). For the remaining standards, there were differences in M_w values, the variation being more pronounced with increasing molar mass.

Table 2
Comparison of weight-average molar mass values ($M_w \times 10^{-3}$ g/mol) for dextran samples determined by HPSEC, AsFlFFF and DOSY.

Sample	DMSO + 0.01 M LiBr	H_2O + 0.1 M $NaNO_3$		D_2O
	HPSEC	HPSEC	AsFlFFF	DOSY
Dx *Leuconostoc* spp.	535	1668	2043	1098
Dx *W. confusa*	1473	6373	6168	23 860
Dx *L. citreum*	1849	11 330	62 800	26 920

3.3. HPSEC and AsFlFFF analyses for HMM dextrans

The molar mass data from the HPSEC and AsFlFFF analyses for the HMM samples are shown in Table 2. The molar mass of commercial *Leuconostoc* spp. dextran obtained with HPSEC and AsFlFFF in aqueous solution was comparable to the value provided by the manufacturer (~2 000 000 g/mol). However, in the HPSEC chromatogram (not shown) and AsFlFFF fractogram (Fig. 1) of this sample, separation of high molar mass components was observed. The HMM components gave a stronger light scattering (LS) signal compared to the main fraction in this sample, which has a lower LS signal but higher RI response (Fig. 1). Integration of the main fraction (the first part of the bimodal RI peak in the AsFlFFF fractogram, Fig. 1) resulted in an M_w value of 500 000 g/mol. Interestingly, this molar mass correlated with the molar mass obtained with HPSEC in DMSO (535 000 g/mol). The molar mass distribution for the commercial *Leuconostoc* spp. dextran obtained with HPSEC in aqueous- and DMSO-based solvents, are shown in Fig. 2. A clear shoulder occurs in the HMM region of the distribution obtained in the aqueous analysis, whereas the distribution from the DMSO analysis is significantly narrower.

The AsFlFFF elution profiles (RI, LS 90°) for the *W. confusa* and *L. citreum* dextrans together with the cross-flow gradient used are presented in Fig. 3. Only one peak in the RI and LS signals eluting at the same retention time can be observed for the *L. citreum* dextran, whereas in the fractogram of the *W. confusa* dextran the LS peak is divided into two. Clearly most of the *W. confusa* dextran elutes between 8 and 15 min according to the concentration-sensitive RI signal. The dextran from *W. confusa* elutes earlier than the dextran from *L. citreum*, which reflects the smaller size of the *W. confusa* dextran than *L. citreum* dextran according to the normal elution mode theory in AsFlFFF. Both dextran samples elute completely during the exponential decay of the cross-flow gradient.

The molar mass and viscometric radius (R_η) data from the aqueous-based HPSEC system are plotted over the RI peak for the dextrans from *W. confusa* and *L. citreum* (Fig. 4). The corresponding

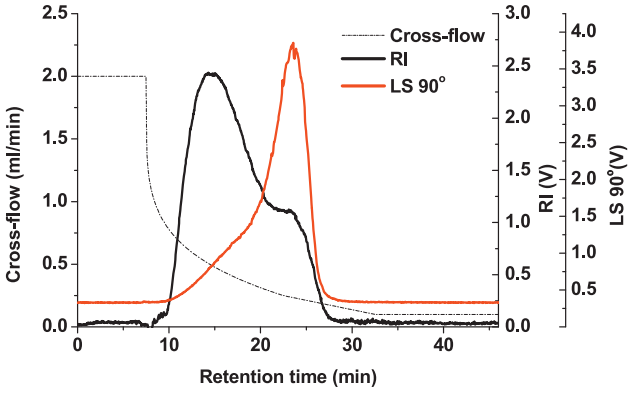

Fig. 1. Refractive index (RI, black line) and light scattering at 90° (LS 90°, red line) profiles, and cross-flow gradient (dotted line) from AsFlFFF for the *Leuconostoc* spp. dextran. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

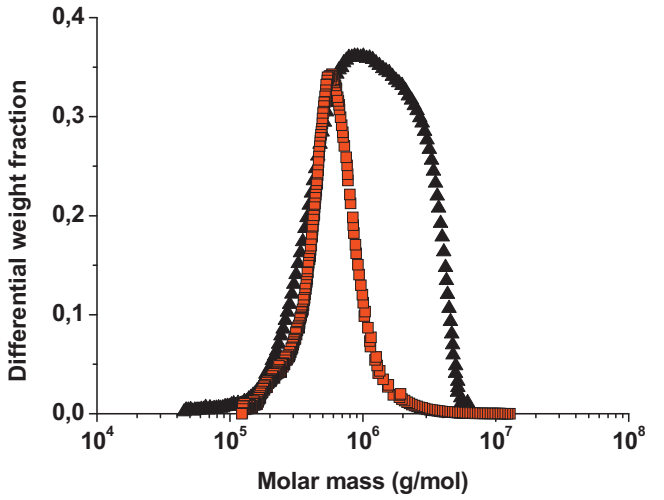

Fig. 2. Molar mass distributions for the dextran from *Leuconostoc* spp. obtained with HPSEC in H_2O + 0.1 M $NaNO_3$ (triangles) and in DMSO + 0.01 M LiBr (red squares). (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

illustration obtained with AsFlFFF with the radius of gyration (R_g) is shown in Fig. 5. Both molar mass and radii are higher for the dextran from *L. citreum* than for the dextran from *W. confusa*. When the aqueous HPSEC and AsFlFFF data are compared (Table 2), the M_w values for the *W. confusa* dextran in aqueous solution are consistent, while the M_w for the *L. citreum* dextran differ significantly. Considering the high molar masses of these dextrans, their R_g values (Fig. 5) are low, indicating a highly compact conformation in

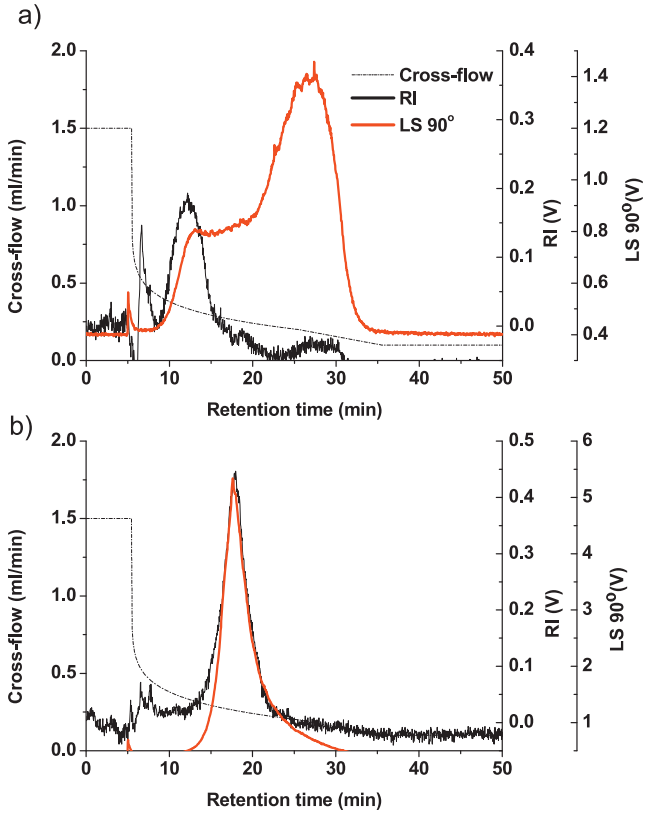

Fig. 3. Refractive index (RI, black line) and light scattering at 90° (LS 90°, red line) profiles, and cross-flow gradient (dotted line) from AsFlFFF for the (a) *W. confusa* and (b) *L. citreum* dextrans. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

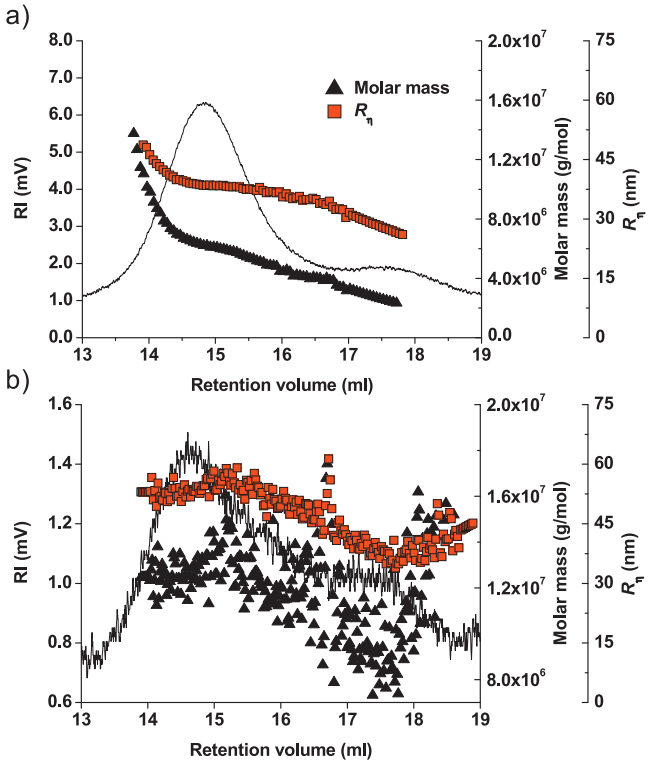

Fig. 4. HPSEC elution profile (RI), molar mass (triangles), and viscometric radius (R_η, red squares) obtained in H_2O + 0.1 M $NaNO_3$ (aq) for the dextrans from (a) *W. confusa* and (b) *L. citreum*. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

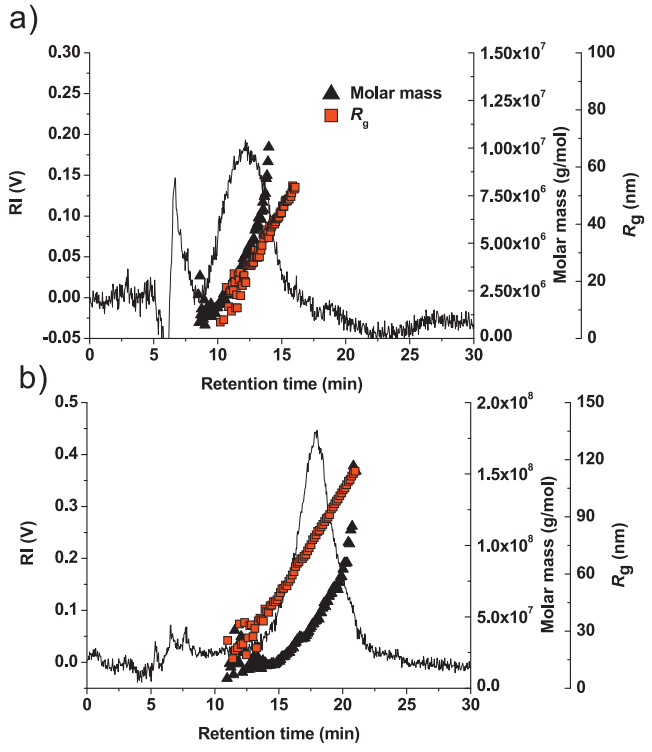

Fig. 5. AsFlFFF elution profile (RI), molar mass (triangles), and radius of gyration (R_g, red squares) for the dextrans from (a) *W. confusa* and (b) *L. citreum*. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

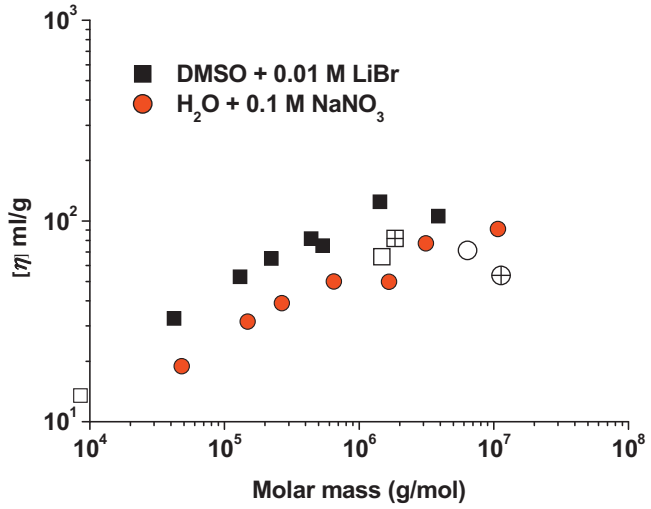

Fig. 6. Relationship between intrinsic viscosity and molar mass of the dextran samples (Mark–Houwink plot). The dextran from *W. confusa* is marked with an open square (DMSO) and open circle (aqueous solution) while the dextran from *L. citreum* is marked with similar symbols that contain a cross. Experimental average molar mass (M_w) and average intrinsic viscosity ([η]) for the standards and HMM samples were used to construct the plot.

the aqueous solution. In DMSO, the molar masses *W. confusa* and *L. citreum* dextrans were significantly lower than in water (Table 2). Unlike the molar masses, the intrinsic viscosities obtained with HPSEC in DMSO for the dextran standards and samples were higher in DMSO than in water (Fig. 6, Table S1.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.021.

3.4. DOSY analysis

DOSY experiments were carried out at 300 K using dilute solutions of the dextran standards and samples. In addition to adjusting the pulse sequence parameters for each sample, other factors that contribute to the reliability of the results were considered. Signal attenuation by convection, due to an apparent temperature gradient along the NMR tube, was observed from the presence of negative peaks in the 1D spectra of the diffusion dataset, especially for HMM dextrans. We therefore utilized the BPPSTE pulse sequence that included convection compensation (CC) to eliminate this effect. Additionally, to further minimize convection effects as well as artifacts from gradient non-linearity, the sample heights were kept below 4 cm. Table 3 shows the D and R_h values for the dextran standards and samples determined from the DOSY data.

Table 3
Diffusion coefficients (D) and hydrodynamic radii (R_h) of the dextran standards and samples determined with DOSY in D_2O at 300 K.

Dextran[a]	Self-diffusion coefficient $D \times 10^{-12}$ ($m^2\ s^{-1}$)	Hydrodynamic radius R_h (nm)
Dx 12	104.7 ± 0.5	2.1
Dx 50	53.7 ± 0.2	4.1
Dx 150	32.6 ± 0.3	6.7
Dx 270	26.9 ± 1.1	8.2
Dx 490	13.9 ± 0.1	11.0
Dx 670	19.2 ± 0.1	15.8
Dx 3500	9.6 ± 0.4	22.9
Dx 11 900	4.3 ± 0.1	51.1
Dx *Leuconostoc* spp.	14.8 ± 0.1	14.9
Dx *W. confusa*	4 ± 0.1	55.0
Dx *L. citreum*	3.8 ± 0.1	57.9

[a] Dx 12 = dextran 12 000 Da.

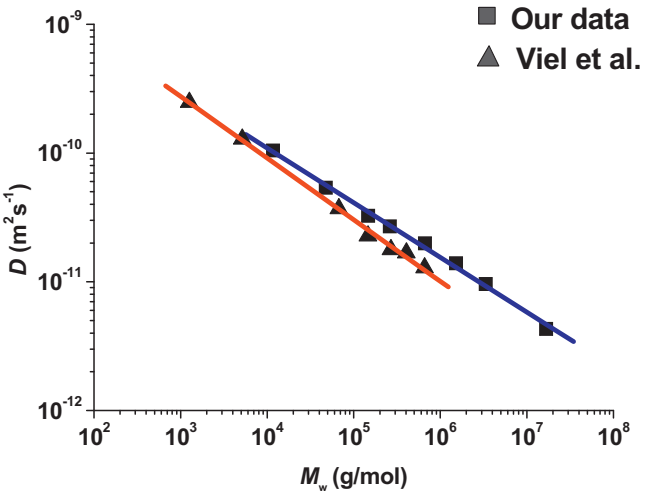

Fig. 7. Double logarithmic plot of self-diffusion coefficient values (D) from the DOSY spectra against the weight average molecular weight (M_w) of the dextran standards. Squares represent our data, obtained with the BPPSTE pulse sequence with convection compensation at 300 K in D_2O. The M_w values measured with light scattering, according to the manufacturer's product sheets (Table 1), were used in the calibration curve. Viel et al.'s data (Viel et al., 2003) (triangles) obtained with the BPPSTE pulse sequence with a longitudinal eddy current delay at 300 K are also shown.

The R_h values were calculated with the Stokes–Einstein equation (3). The D value for residual water (HDO) was the same for all the samples ($\sim 2.2 \times 10^{-9}$ m^2 s^{-1}).

Fig. 7 shows a calibration curve for the DOSY data (double logarithmic plot of D against M_w), according to the method of Viel et al. (2003). Fig. 7 also shows Viel et al.'s data for dextran standards with a lower molar mass range (1270 to 668 000 g/mol). In Fig. 7, the M_w values from the dextran standards measured with light scattering, as specified by the manufacturer, were used since they gave the best calibration curve ($R^2 = 0.996$). For example, the M_w of Dx 490 (490 000 g/mol based on calibration with pullulan standards) distorted the calibration curve, while the value obtained with light scattering (1 520 000 g/mol) gave a better fit. From the curve, the scaling parameters K and α (Eq. (2)) were 5.47×10^{-9} and -0.425, respectively ($D = 5.47 \times 10^{-9} \times M_w^{-0.425}$).

A comparison of the M_w values obtained with AsFlFFF, HPSEC, and DOSY is shown in Table 2. An example of the DOSY spectra obtained with NMRnotebook is shown in Fig. 8. The figure shows overlaid DOSY spectra from the dextran standards with nominal M_w of 150 000 g/mol (Dx 150), 670 000 g/mol (Dx 670) and

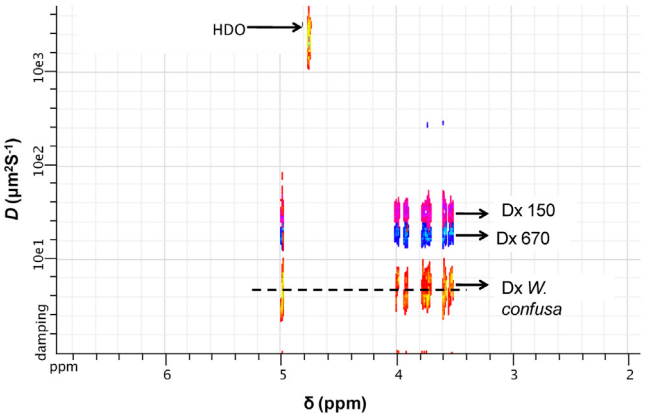

Fig. 8. Overlaid DOSY spectra of the dextran standards with nominal molar mass of 150 000 g/mol (Dx 150), 668 000 g/mol (Dx 670) and the dextran produced by *W. confusa*. The DOSY data were measured at 300 K in D_2O using the BPPSTE pulse sequence with convection compensation.

W. confusa dextran. The peaks of the dextran standards had a more narrow width in the diffusion dimension than the peaks of the dextran isolated from *W. confusa* (Fig. 8). This was most probably due to the molecular dispersity of the isolated dextran sample.

4. Discussion

In this study, the macromolecular properties of several dextran standards and HMM dextran samples produced by *Leuconostoc* spp., *W. confusa* and *L. citreum* were determined using three methods (HPSEC, AsFlFFF and DOSY NMR) and two different solvents (DMSO and water).

All dextrans analyzed in this study were class 1 dextrans. The dextran standards, commercial dextrans from *Leuconostoc* spp. had less than 5% α-(1→3) branch linkages according to NMR integrations (not shown). *W. confusa* dextran contains 2.7% α-(1→3) branch linkages while *L. citreum* dextran contains 3.5% α-(1→3) and 11% α-(1→2) branch linkages (Maina et al., 2008). As previously shown, even with few branch linkages, some of the branches in *W. confusa* dextran are elongated by two or more α-(1→6)-linked glycosyl units (Maina et al., 2011).

Excluding *L. citreum* dextran, the study showed that, for aqueous solutions, HPSEC and AsFlFFF gave relatively similar results for dextran standards and samples (Tables 1 and 2). Furthermore, the values obtained with these methods for the dextran standards and HMM commercial *L. mesenteroides* spp. dextran, were comparable to the values reported by the manufacture using HPSEC. On the contrary, the M_w values obtained in DMSO were only comparable until Dx 270 (Table 1) after which clear variation was observed especially for the HMM samples. The discrepancies in molar mass values in DMSO and aqueous solution may result from an interplay of several factors discussed in detail in following sections.

4.1. Solubility and aggregation

When considering solubility, the main concerns are (a) whether solubilized material is representative of the sample analyzed, (b) is the solubilized portion similar in both solvents and consequently, and (c) are the values obtained with both solvents directly comparable. The solubility of the LMM dextrans standards (Dx12–Dx 670) and the commercial *L. mesenteroides* dextran was above 80% in both solvents which is sufficient to presume that the solutions were representative of the whole sample and can be compared. For the HMM dextrans however, the recovery was low and thus the solutions analyzed only represented the soluble fractions of the samples. As shown in Tables 1 and 2, there was satisfactory agreement for Dx12–Dx 270 while for Dx 490, Dx 670 and especially commercial *L. mesenteroides* dextran, there were differences in the molar values obtained in DMSO and aqueous solutions. We can therefore postulate that the difference in molar mass values of the latter dextrans arises from differences in the state of the dextran molecules in the solvents, most likely due to the presence of aggregates in the aqueous solutions. The significant differences in molar mass values of the HMM dextrans was indicative of a higher aggregation tendency as the molar mass of the dextran increased.

Aggregation of dextrans in dilute solutions has not been significantly emphasized in literature. It is common in polysaccharides solutions and occurs due to inter- and intra-molecular hydrogen bonds. Organic solvents such as DMSO disrupt the hydrogen bonds and are widely used for dissolution of other α-glucans, namely amylose, amylopectin and glycogen (Gilbert, 2011; Schmitz, Dona, Castignolles, Gilbert, & Gaborieau, 2009). As Gidley et al. maintain (2010), aggregates cause incorrect apparent size distributions. This effect is clearly observed with commercial dextran from *Leuconostoc* spp. The monomodal molar mass distribution (Fig. 1) and M_w

(Table 2) obtained with HPSEC indicates that the DMSO solution of this sample was mainly composed of individual chains with an M_w of 535 000 g/mol. As seen in the AsFlFFF fractogram (Fig. 2) of this same sample, both the RI and LS peaks are bimodal indicating the presence of two populations in aqueous solution, which we considered to be individual chain and aggregates. The M_w (500 000 g/mol) for the first, more abundant population, which most probably consists of individual chains, was the same as for the sample dissolved in DMSO and analyzed with HPSEC. When the whole AsFlFFF peak is considered, the presence of aggregates significantly skews the molar mass value (2 043 000 g/mol) since the aggregates dominate the light scattering signal. Previously, a similar bimodal behavior of commercial dextran from *Leuconostoc* spp. in aqueous HPSEC was observed by Ioan et al. (2000). Nonetheless, they considered it to result from poor resolution in HPSEC instead of the presence of aggregates in aqueous solution, which is proposed here.

The low solubility of the HMM dextran standards and samples from *W. confusa* and *L. citreum* impedes direct comparison of the molar mass in water and DMSO. Nonetheless, the presence of aggregates in the aqueous solutions of these samples largely explains the differences in molar mass values in the two solvents. Separation of a very low amount of large aggregates in the *W. confusa* dextran was observed in AsFlFFF (Fig. 3). However, the apparent molar mass value of this dextran in aqueous solution, and also that of *L. citreum* dextran, was significantly higher than the values obtained in DMSO (Table 2). Thus the extent of aggregation in these samples cannot be deduced from these results, either the whole sample in aqueous solutions eluting as one peak (Figs. 4 and 5) is aggregated or the samples contain compact aggregates that co-elute with individual chains.

4.2. Chain degradation or disaggregation

Differences in the aqueous and DMSO data may also result from chain degradation during HPSEC analysis in DMSO solution (Cave, Seabrook, Gidley, & Robert, 2009; Striegel, Isenberg, & Cotê, 2009). However, we analyzed the *W. confusa* dextran with off-line DLS in DMSO, and no significant difference between the number-average R_h from DLS (22 nm) and the R_η from HPSEC (24 nm) was observed, confirming that the degradation of the sample was not significant. Although studies have attributed the degradation (breaking of covalent bonds) of HMM polysaccharides during HPSEC analysis in DMSO owing to shear forces (Cave et al., 2009; Striegel et al., 2009), disruption of aggregates due to the same process in either DMSO or aqueous solutions should also be considered. These two distinct processes (degradation and disaggregation) are, however, difficult to distinguish and may be confused with one another, when analyzing molecularly disperse HMM samples. In fact, the discrepancy in the molar masses of the *L. citreum* dextran in aqueous AsFlFFF and HPSEC may result from a certain degree of disaggregation of the sample in the HPSEC analysis. Vice versa, a lack of substantial shear forces to disentangle aggregates explains the higher M_w value in the AsFlFFF analysis, where the sample is separated in an open channel.

4.3. Dextrans occupy larger space in DMSO than in aqueous solution

Higher values for the intrinsic viscosity of dextrans were obtained in DMSO than in water. Similar results have also been reported by Alekseeva, Eliseeva, Noskov, and Rozhkova (2007), who compared the intrinsic viscosities of dextrans in water, formamide, and dimethyl sulfoxide (DMSO), and found that intrinsic viscosities were highest in DMSO and lowest in water. The researchers also concluded that dextran molecules swell in DMSO, which promotes the dissolution of the polymer. Similar observation on the expansion of dextrans in DMSO was reported by Merienne, Busnel, Fricoteaux, and Prudhomme (2000), who measured the average values for radii (R_g, R_h) using HPSEC in water and DMSO with different salt addition. The values were higher in DMSO than in water, indicating more expanded conformation of dextrans in DMSO.

In general, regardless of the solvent used, the Mark–Houwink plots (Fig. 6) start to curve (i.e., the relationship deviates from the expected linear behavior) at the higher molar masses. This has been attributed to the increased branching density (branching units per macromolecule) and the length of the branches (Ioan et al., 2000; Kasaai, 2012; Nordmeier, 1993). However, the presence of compact aggregates in the aqueous solutions of the *W. confusa* and *L. citreum* dextrans may also lower the intrinsic viscosity values obtained with aqueous HPSEC and hence cause the non-linearity of the Mark–Houwink plot. The lowered values for intrinsic viscosity and further non-linearity of the Mark–Houwink plot of corncob arabinoglucuronoxylan have been explained by the presence of compact supramolecular aggregates (Dhami, Harding, Elizabeth, & Ebringerovà, 1996).

4.4. Applicability of DOSY

Dilute solutions were used in the DOSY experiments to prevent entanglement of the dextran chains that leads to inaccurate D values. According to Antalek (2002), the artifacts and erroneous results in the DOSY spectra can arise from eddy currents, gradient field non-uniformity, and convection currents. Several pulse sequence modifications and measurement setups to minimize their effects have been proposed (Antalek, 2002). The effects of gradient field non-uniformity and convection currents can be reduced by restricting the sample volume to the section of the probe with the most uniform gradient field and efficient temperature control (Antalek, 2002). Though the sample height was restricted to ~4 cm in this study, utilizing a pulse sequence with convection compensation was the only way to truly eliminate artifacts from convection currents.

The molar mass range of dextran standards (12 000 to 11 900 000 g/mol) utilized in constructing a calibration curve for the DOSY data in this study, was significantly larger than has been used in previous studies (Politi et al., 2006; Viel et al., 2003). Nonetheless, a reliable calibration curve was obtained ($R^2 = 0.996$). One limitation of DOSY is the need to have reliable molar mass values for the standards used in the calibration curve. In this study, the M_w values specified by the manufacturer based on light scattering (Table 1) gave a better curve compared to data obtained with calibration standards. The scaling parameters obtained in this study, $K = 5.47 \times 10^{-9}$ m^2 s^{-1} and $\alpha = -0.425$, were different from values obtained previously by Viel et al. (2003) using pullulan standards ($K = 8.2 \times 10^{-9}$ m^2 s^{-1} and $\alpha = -0.49$) and dextran standards (1270–668 000 g/mol, $K = 7.61 \times 10^{-9}$ m^2 s^{-1} and $\alpha = -0.478$). However, Politi et al. (2006) found $K = 4.24 \times 10^{-9}$ m^2 s^{-1} and $\alpha = -0.417$ (note, in the original article K is given as $\text{Log}\,K = -8.372$ and the experiments were carried out at 298 K) with dextran standards ranging from 10 000 to 500 000 g/mol, which are in agreement with the values obtained here. As shown in Fig. 7, data from this study are reasonably parallel to those of Viel et al. (2003), obtained with a BPPSTE pulse sequence that included a longitudinal eddy current delay. This suggests that the difference in the scaling parameters may result from instrumental errors. Furthermore, it underscores the need to exercise caution when using scaling parameters reported in the literature to calculate the M_w from DOSY data obtained with a different instrument or pulse sequence.

The samples used in DOSY analysis most likely contain aggregates which remain intact since they do not experience any shear forces during the analysis. Thus in addition to the molecular dispersity of the HMM dextrans the aggregates contribute to the

distribution of D values obtained in DOSY. Callaghan and Pinder proposed that D values in DOSY are molar mass weighted (D_w) (Callaghan & Pinder, 1983). The molar mass of *Leuconostoc* spp dextran was lower (Table 2) than the value obtained in both HPSEC and AsFlFFF. This may however result from the presence of a significant amount of molecules with a molar mass of 500 000 g/mol in aqueous solution as shown with AsFlFFF analysis (Fig. 2). Considering the lower molar mass of *W. confusa* and *L. citreum* dextrans in DMSO (Table 2), no significant peaks were observed in the DOSY spectra at higher D values, indicating that the aqueous solutions in DOSY predominantly contained aggregated dextran molecules. The higher molar mass values for the dextrans in the DOSY analysis may result from formation of larger aggregates in the D_2O sample solutions since they did not contain salts. Nonetheless, the order of magnitude ($\sim 10^7$) of the values (Table 2) obtained with DOSY, were in satisfactory agreement with the HPSEC and AsFlFFF values. Therefore, as previously proposed (Viel et al., 2003), DOSY analysis can be used to estimate the size of dextrans in aqueous solution even at HMM as shown in this study.

A varying range of R_h values are reported in the literature (Armstrong, Wenby, Meiselman, & Fisher, 2004; Ioan et al., 2000; Nordmeier, 1993) for dextrans of different sizes. The R_h values calculated from the DOSY data in this study were generally lower than the values reported previously for dextrans with similar molar mass. For example, the R_h of the 2 000 000 g/mol *Leuconostoc* spp. dextran was 14.9 nm from the DOSY data, while Armstrong et al. (2004) and Ioan et al. (2000) reported values of 26.9 nm and 47.0 nm, respectively, for similar dextrans. Since these studies utilize aqueous solutions, the difference in the measured R_h values may be attributed not only to methodological factors but also to the presence of aggregates in the solutions analyzed.

5. Conclusions

In this study, the molar mass values of HMM dextran standards, a commercial dextran from *Leuconostoc* spp., and dextrans produced by *W. confusa* and *L. citreum*, from HPSEC, AsFlFFF and DOSY NMR were higher in aqueous solution in comparison with molar masses obtained with HPSEC in DMSO solution. This was attributed to the presence of aggregates in aqueous solution that were less prevalent in DMSO. This difference between molar masses in different solvents should be taken into consideration when using dextran standards for HPSEC calibration. This study showed that all of these methods can be used for estimating the size of HMM dextrans. However, DMSO is the solvent of choice when the objective is to determine the size of individual dextran chains, for example, when comparing samples produced by different strains or evaluating the effect of cultivation conditions on the molar mass of the dextrans produced. To get accurate molar mass data for individual dextran molecules, obtaining aqueous solutions that do not contain aggregates would be important. It might be challenging, however, to prepare aggregate-free aqueous solutions without causing chain degradation. Nonetheless, food matrixes such as sourdough are water-based systems; it is therefore necessary to also consider whether the functionality of dextrans is correlated to the molar mass of individual molecules or the aggregates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.021.

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