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Short communication

Improved reproducibility in the determination of the molecular weight of chitosan by analytical size exclusion chromatography

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ABSTRACT

The reproducibility of the determination of the molecular weight of chitosans in the 90–210 kDa range (M_n) by analytical size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS) was improved by reducing the salt concentration in the mobile phase from (0.3 M acetic acid, 0.2 M sodium acetate, and 0.8 mM sodium azide) to (0.15 M acetic acid, 0.1 M sodium acetate, and 0.4 mM sodium azide) using Tosoh TSKgel G6000PW$_{XL}$ and G5000PW$_{XL}$ columns in series. The variability of measured molecular weight was significantly reduced by lowering the acetate concentration in the mobile phase, while the average molecular weight did not change significantly. The coefficient of variation of the number-average molecular weight, CV(M_n), decreased from 7–12% to 3–6% upon mobile phase dilution. This reduced variability in molecular weight of chitosans obtained from SEC is a significant improvement when precise values of chitosan molecular weight are required, for example in stability studies where viscosity changes in concentrated chitosan solutions are assessed, and in gene delivery applications.

1. Introduction

Chitosan, a natural copolymer of amino-glucopyranosyl and *N*-acetyl-glucopyranosyl, has found numerous applications in bioengineering, drug delivery, and gene therapy. The physicochemical, biological, and rheological properties of this polymer vary significantly as a function of its molecular weight and molecular weight distribution (Beri, Walker, Reese, & Rollings, 1993; Denuzière, Yagoubi, Baillet, & Ferrier, 1995). It is therefore important, and in some cases critical, to know precise and accurate values of the molecular weight of chitosan. It is well known that the determination of the molecular weight of polyelectrolytes is complex (Terbojevich, Cosani, Focher, & Marsano, 1993). In the case of chitosan, this situation is exacerbated due to the marked tendency of this polymer to form resilient aggregates in solution (Anthonsen, Varum, Hermansson, Smidsrod, & Brant, 1994; Liu & Yao, 2002; Philippova, Volkov, Sitnikova, & Khokhlov, 2001; Schatz, Viton, Delair, Pichot, & Domard, 2003b).

The most direct method for molecular weight characterization is aqueous size exclusion chromatography, SEC (or gel filtration chromatography). SEC provides the number-average molecular weight, M_n, and the weight-average molecular weight, M_w, and therefore the polydispersity index M_w/M_n in a single measurement. It has been used extensively in studies of chitosans, with the first SEC characterization of chitosan being reported by Wu and coworkers in 1976, who devised a protocol involving injection of chitosan solutions (5–10 mg/mL) into a column combination of 18 ft total length packed with glycerol-coated glass particles eluted with a 2% acetic acid solution. Refractive index, RI, and ultra-violet absorption, UV, detection were employed and molecular weights were determined relative to dextran standards (Wu, Bough, Conrad, & Alden, 1976). Other chitosan SEC methods studies have used single detectors, mainly refractometers (Knaul, Kasaai, Bui, & Creber, 1998; Li, Du, & Xu, 2004; Liu, Du, Wang, Hu, & Kennedy, 2004; Muzzarelli, Tosi, Francescangeli, & Muzzarelli, 2003; Muzzarelli, Xia, Tomasetti, & Ilari, 1995; Prashanth & Tharanathan, 2005; Terbojevich et al., 1993; Tsaih & Chen, 1999). This approach requires calibration that relates elution time to molecular weight via narrow polydispersity standards of known molecular weight. Unfortunately this type of calibration only provides molecular weights relative to the standards, such that accuracy depends on the assumption of a similar solution behavior of chitosan to the chosen standard (Beri et al., 1993), namely dextran (Prashanth & Tharanathan, 2005; Wu et al., 1976) pullulan (Chen, Du, Tian, & Sun, 2005; Chen, Du, & Zeng, 2003; Knaul et al., 1998), or polyethylene oxide (Kato, Onishi, & Machida, 2002; Le, Tran, Naotsugu, Fumio, & Tamikazu, 2003). This is not a valid assumption and

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therefore generally results in significantly overestimated molecular weights (Beri et al., 1993; Ottoy, Varum, Christensen, Anthonsen, & Smidsrod, 1996). Terbojevich and coworkers addressed this issue by calibrating their SEC system with broad polydispersity chitosans of different molecular weights, using M_w results from batch static light scattering and theoretical polydispersity ratios (Terbojevich et al., 1993), however such standards are generally unavailable.

Light scattering detectors have been adapted to SEC to detect size and are used in combination with refractometers or UV detectors, which serve as concentration detectors. This technique generates absolute molecular weights and additional molecular size data, such as the radius of gyration and the hydrodynamic radius, depending on the specific detection system. Several types of laser light scattering detectors have been used in chitosan SEC methods, generally combined with refractometers as concentration detectors, including (i) right angle laser light scattering, to which a four-capillary differential viscometer was added for intrinsic viscosity and size information (Lavertu, Méthot, Tran-Khanh, & Buschmann, 2006), (ii) low angle laser light scattering (Christensen et al., 1996; Kubota, Tatsumoto, Sano, & Toya, 2000; Ottoy et al., 1996; Rinaudo, Milas, & Le Dung, 1993) and (iii) multi-angle laser light scattering, MALLS, which has been the most popular light scattering detector to date (Beri et al., 1993; Brugnerotto, Desbrières, Heux, Mazeau, & Rinaudo, 2001a; Brugnerotto, Desbrières, Roberts, & Rinaudo, 2001b; Fee et al., 2003; Fernandez-Megia, Novoa-Carballal, Quiñoá, & Riguera, 2005; Jiang et al., 2006; Köping-Höggård et al., 2004; Kujawa, Moraille, Sanchez, Badia, & Winnik, 2005; Lamarque, Lucas, Viton, & Domard, 2005; Piron & Domard, 1998; Rinaudo et al., 1993; Schatz, Pichot, Delair, Viton, & Domard, 2003a; Sorlier, Denuzière, Viton, & Domard, 2001; Tommeraas et al., 2002; Yanagisawa, Kato, Yoshida, & Isogai, 2006).

Current SEC methods are listed in Table 1 and have used a number of different combinations of columns, mainly PW and PW_{XL} columns from Tosoh Bioscience, acetate buffer mobile phases, generally at a pH near 4.5, and chitosan concentrations between 0.02 and 2 mg/mL, with up to 10 mg/mL for low molecular weight chitosans.

The need for precise measurements of chitosan molecular weight arose while investigating the long term storage stability of chitosan solutions. Viscosity measurements were routinely used to assess changes in chitosan solutions properties due to chitosan hydrolysis, precipitation or gelation and we required accurate values of chitosan molecular weights in these solutions in order to interpret viscosity changes. Since we were measuring viscosity changes on the order of 50% and the viscosity of a polymer solution in concentrated systems is approximately proportional to the cube of the molecular weight of the polymer (Boris & Colby, 1998; Dobrynin, Colby, & Rubinstein, 1995; Rubinstein, Colby, & Dobrynin, 1994), we required a method for determining the molecular weight of chitosan with as low variability as possible. Errors in molecular weight assessment due to lack of precision or variability were the limiting factor of these analyses (a 20% decrease in molecular weight can induce a 50% decrease in viscosity in concentrated solutions) and it became necessary to scrutinize factors in SEC analysis that could influence the precision and variability of the molecular weight data.

One parameter that can affect precision and variability of chitosan SEC analyses is the ionic strength of the SEC mobile phase. It should be sufficiently high to screen the protonated amine groups on chitosan (Berth, Dautzenberg, & Peter, 1998) and to avoid irreversible adsorption on columns (Ottoy et al., 1996; Terbojevich et al., 1993) and ion-exclusion effects (Terbojevich et al., 1993). However, for chitosan at 1 mg/mL, which is a typical concentration in SEC, the amine concentration is only on the order of 5 mM, depending slightly on the degree of deacetylation (DDA). There is therefore a large salt excess in typical SEC buffers that can not only screen electrostatic repulsion, but possibly alter folding, aggregation and solubility of the fully charged polymer chains in solution, and thereby elution properties. Consequently, the mobile phase salt concentration could impact significantly the precision and variability of chitosan characterization by SEC. We undertook a study to assess the reproducibility and variability of molecular weight analysis of chitosans by analytical SEC-MALLS as a function of the salt concentration in a mobile phase composed of acetic acid and sodium acetate and report here the results of this investigation. We also provide guidelines for optimal SEC conditions which will be of practical use to scientists who employ chitosan to design biomaterials, gene delivery systems, and for other applications.

2. Materials and methods

2.1. Chemicals

Acetic acid (EMD, glacial, 99.7%), sodium acetate (Sigma–Aldrich, ACS reagent, 99+%), sodium azide (Sigma–Aldrich, 99%),

Table 1
Current SEC methods used for molecular weight analysis of chitosan: columns, mobile phases, and sample concentrations, [chitosan]

SEC column	PW and PW_{XL} columns (Tosoh Bioscience) - Linear column $GMPW_{XL}$ (for wide molecular weight range) (Knaul et al., 1998; Kubota et al., 2000; Kujawa et al., 2005; Lavertu et al., 2006; Muzzarelli et al., 1995) - Single column G5000PW (Li et al., 2004; Liu et al., 2004) - Two-column set, *e.g.* $G3000PW_{XL}$-$G6000PW_{XL}$ (Lamarque et al., 2005), $G4000PW_{XL}$-$G5000PW_{XL}$ (Tsaih & Chen, 1999), G6000PW-G5000PW (Ottoy et al., 1996) - Three-column set: $G6000PW_{XL}$-$G5000PW_{XL}$-$G4000PW_{XL}$ (Tommeraas et al., 2002), G5000PW-G4000PW-G3000PW (Fee et al., 2003) Shodex columns o OHpak B 803 and 805 (Rinaudo et al., 1993) o SB-806 M (Jiang et al., 2006; Yanagisawa et al., 2006) Two-column combination of Eichrom MICRA-Gold CATSEC 100 and 1000 (Brugnerotto et al., 2001a, 2001b) PSS Novema GPC column 10 μ combined with PSS Novema 3000 column (Fernandez-Megia et al., 2005) Pharmacia HR 10/30 column with in-house prepared poly(styrene-*co*-divinylbenzene) packing (Christensen et al., 1996)
Mobile phase and [chitosan]	Acetic acid (AcOH) and sodium acetate (NaOAc) o 0.3 M AcOH/0.2 M NaOAc (Brugnerotto et al., 2001a, 2001b; Kujawa et al., 2005; Lavertu et al., 2006; Rinaudo et al., 1993), [chitosan] = 0.5 mg/mL (Brugnerotto et al., 2001a, 2001b), or 1.0 mg/mL (Kujawa et al., 2005; Lavertu et al., 2006) o 0.2 M AcOH/0.1 M NaOAc (Li et al., 2004; Liu et al., 2004; Tsaih & Chen, 1999), [chitosan] = 1 mg/mL (Tsaih & Chen, 1999) or 0.5 mg/mL (Liu et al., 2004) Acetic acid and ammonium acetate (NH_4OAc) o 0.2 M NH_4OAc, [chitosan] = 0.02–2 mg/mL (Christensen et al., 1996), 0.125–0.250 mg/mL (Ottoy et al., 1996), or 1 mg/mL (Tommeraas et al., 2002) o 0.2 M AcOH/0.15 M NH_4OAc, [chitosan] = 0.4–1 mg/mL (Lamarque et al., 2005), 1 mg/mL (Fernandez-Megia et al., 2005; Schatz et al., 2003a; Sorlier et al., 2001) or 4 mg/mL (for lower molecular weight samples) (Schatz et al., 2003a) 0.5 M acetic acid/0.1 M sodium nitrate, [chitosan] = 0.5–2 mg/mL (Yanagisawa et al., 2006)

and double deionized water were used for mobile phase preparation. Double deionized water, ddH_2O, was obtained by filtration of deionized water with a milli-Q system: gradient 10, equipped with a Quantum™ EX Ultrapure Organex cartridge and a Millipak® Express 20 filter with 0.22 μm of pore size (Millipore). All solutions for mobile phase preparation were filtered before use with a hydrophilic Durapore® membrane with 0.1 μm pore size and 47 mm in diameter (Millipore product # VVLP 04700). Toluene (HPLC grade, Fisher) and sodium chloride, NaCl (Sigma, ACS reagent, ⩾99.0%) were used for the calibration of the detectors DAWN EOS and Optilab DSP, respectively.

2.2. Chitosans

The chitosans studied are shown in Table 2, along with their DDAs, determined by proton nuclear magnetic resonance spectroscopy, ^{1}H NMR (Lavertu et al., 2003), and nominal molecular weights, obtained by analytical SEC in the half-diluted buffer. Chitosans were obtained from Bio Syntech Canada Inc. (Laval, QC, Canada), and Wako Chemicals USA (Richmond, VA, USA). They were used as received or converted to lower molecular weight samples, with a previously described procedure using nitrous acid (Lavertu et al., 2006).

2.3. SEC-MALLS

The system was composed of an Agilent 1100 Series Chromatography system, with a micro-vacuum degasser (Agilent G1379A), an isocratic pump (Agilent G1310A). An inline filter (Wyatt Technology Corporation) was installed between the pump and the autosampler (Agilent G1329A), to remove any particle from the mobile phase, the micro-vacuum degasser, or the pump. It was equipped with a hydrophilic Durapore® membrane of pore size 0.1 μm and 25 mm in diameter (Millipore). The detectors were a MALLS DAWN EOS calibrated with toluene, and a differential refractometer Optilab DSP, calibrated with aqueous sodium chloride. Both detectors were manufactured by Wyatt Technology Corporation, and functioned at 690 nm. The DAWN EOS was normalized with a nearly monodisperse pullulan standard P-5 (5.00 mg/mL, Shodex, standard kit P-82), with M_w and polydispersity index (PDI) of 5900 Da and 1.07, respectively, and dissolved in the acetate buffer used as SEC mobile phase.

2.4. Column configurations and mobile phase in SEC

Two TSKgel columns ($G6000PW_{XL}$ and $G5000PW_{XL}$, I.D.: 7.8 mm, length: 30 cm, Tosoh Bioscience, Montgomeryville, PA, USA) were used in series, along with a PW_{XL} guard column. The mobile phases consisted of acetate buffers at two different ionic strengths: regular buffer composed of (0.3 M acetic acid, 0.2 M sodium acetate, and 0.8 mM sodium azide), the latter to avoid microbial growth, in double deionized water, and half-diluted acetate buffer (0.15 M acetic acid, 0.1 M sodium acetate, and 0.4 mM sodium azide), corresponding to total acetate concentrations of 0.501 M and 0.250 M, respectively. The mobile phases were filtered through a 0.1 μm hydrophilic Durapore, PVDF, membrane. The diluted buffer was obtained by mixing equal volumes of filtered regular buffer and filtered double deionized water. The pH of the buffer solutions was measured with a pH-meter (Corning, model 320) equipped with a semi-micro electrode (Orion, model 8115 BN) and calibrated with two buffer solutions, at pH 4.00 and 7.00 (Ricca Chemical Company). The pH of the different batches prepared were 4.47–4.51 for both buffers. The conductivity of the buffer solutions was determined using a conductivity meter (Accumet, model 20) with a glass body electrode of conductivity cell constant of 10.0 cm^{-1} (Accumet) calibrated with a standard solution of conductivity 10.097 mS/cm (traceable conductivity calibration standard VWR # 23226-625). The conductivity was 13.4 mS/cm for the regular buffer and 7.61 mS/cm for the half-diluted buffer.

Table 2
Chitosan samples used, and their corresponding labels: T-DDA%-M_n, with T being the type of chitosan, DDA%, the nominal degree of deacetylation and M_n the number-average molecular weight

Chitosan code	DDA (%)	M_n (kDa)	Average dn/dc (mL/g)
B-77-190	77.1	190	0.194
B-81-95	81.2	95	0.190
B-92-205	91.7	205	0.202
W-81-135	80.7	135	0.185
N-86-10	85.6	10	0.191

Type is B for products from Bio Syntech Canada Inc., W for products supplied by Wako Chemicals USA, and N for nitrous acid-degraded Bio Syntech Canada Inc. products. Average dn/dc values were obtained for chitosans in half-diluted buffer at 690 nm.

2.5. Specific refractive index, dn/dc, measurements

The dn/dc values of chitosan solutions were determined in half-diluted buffer at 690 nm. Five chitosan samples spanning high to low molecular weights were used (Table 2), since dn/dc can vary with molecular weight (Itakura et al., 2004; Podešva, Procházka, & Medin, 1995). Polymers were vacuum-dried at ca. 65 °C for 1 day, then stock solutions (0.70 mg/mL) in half-diluted buffer were prepared in duplicates (weighed separately) by direct dissolution and shaking using a LabQuake shaker (Barnstead Thermolyne, model 4002110) at room temperature for two days. Solutions for analysis were obtained by dilution of the stock solutions in half-diluted buffer of different proportions, to provide solutions of concentration 0.70, 0.60, 0.50, 0.40, 0.30, 0.20, and 0.10 mg/mL. Most solutions were clear, and were not filtered prior to injection. Samples prepared with chitosan W-81-135 were not clear; they were filtered prior to analysis using a syringe filter containing a hydrophilic Durapore® membrane of 0.45 μm pore size and 13 mm in diameter (Millipore). The set-up for dn/dc measurements was composed of a micro-vacuum degasser (Agilent G1379A), an isocratic pump (Agilent G1310A), a manual injector (Wyatt injection system) with a 1.0 mL sample loop, and a differential refractometer Optilab DSP, (Wyatt Technology Corporation), with a flow rate of 0.8 mL/min. Values of dn/dc of chitosans B-77-190, B-92-205, B-81-95, W-81-135, and N-86-10 were determined in half-diluted buffer at 690 nm (Table 2). The overall average of dn/dc was 0.192 mL/g with a coefficient of variation of 3.2% and without evident dependence on DDA or molecular weight.

2.6. Analytical SEC

For all samples, chitosan solutions (1.0 mg/mL) were prepared by direct dissolution in either acetate buffer by shaking using a LabQuake shaker at room temperature for two days. The acetate buffers used for dissolution were from the same batch as the mobile phase used for SEC. The samples were filtered with a syringe filter containing a hydrophilic Durapore® membrane of 0.45 μm pore size and 13 mm in diameter (Millipore). Analyses were performed at room temperature with the buffer solution as mobile phase, at a flow rate of 0.8 mL/min, with sample injection volume of 100 μL. SEC data were recorded and analyzed with Astra V software (Wyatt Technology Corporation).

2.7. ^{1}H NMR spectroscopy

Solutions of chitosan (10 mg) in D_2O and DCl (2.00 mL, 0.99:0.01 v:v) were prepared at room temperature. Each sample

was allowed to dissolve for ca. two days with shaking using a LabQuake shaker; 750–1000 μL of the sample solution was then transferred to an NMR tube (Wilmad, 535-PP, 8" long). Spectra were obtained with a Varian Mercury 400 MHz spectrometer at 70 °C according to the procedure described by Lavertu et al. (Lavertu et al., 2003).

2.8. Statistical analyses

Student's *T*-test and the *F*-test were applied to the eight to nine replicate M_n and M_w values found for each of the four different chitosans tested (B-77-190, B-81-95, B-92-205, and W-81-135) in both salt concentrations in analytical SEC, to determine whether means (*T*-test) or variances (*F*-test) of M_n and M_w changed in a statistically significant manner due to the salt concentration change in the mobile phases. *P*-values of the *T*-test and the *F*-test less than 0.05 were considered to indicate significant differences between means or variances of the two groups, respectively.

3. Results and discussion

Acetate buffers have been widely used as SEC mobile phases for chitosan molecular weight characterization (Table 1). In order to determine if the variability of chitosan molecular weight obtained by SEC can be improved by modifying the ionic strength of the mobile phase, our current SEC mobile phase (0.3 M acetic acid, 0.2 M sodium acetate, and 0.8 mM sodium azide) was diluted. In preliminary experiments (not shown), dynamic light scattering in batch mode was performed on this buffer and after dilution to 75%, 50%, and 25% of its original concentration. We noticed higher apparent variability in results for the undiluted buffer than for the dilutions where the 50%-diluted buffer appeared to be the least variable. We therefore chose the half-diluted buffer (50% the original concentration) for a more extensive analysis with multiple replicates in SEC.

Chitosans B-77-190, B-81-95, B-92-205, and W-81-135 were dissolved in each acetate buffer and analyzed using the column set guard PW_{XL}-G6000PW_{XL}-G5000PW_{XL}, in eight, or more, replicates. Monomodal profiles on SEC traces were obtained for all samples, with B-92-205 as a representative example shown in Fig. 1.

RI traces of the five replicates in the half-diluted buffer often superimposed more closely than in the regular buffer (Fig. 1), particularly in the low molecular weight region, suggesting higher variability in RI detection of elution times in the regular buffer compared to half-diluted buffer. Peak widths were determined both at the mid-height of the peak and at its base, the latter corresponding to the time between the defined peak limits (range of elution time of the polymer peak). We found that the mean peak width at mid-height increased slightly (from 4.31 to 4.55 min for the RI signal) when the acetate buffer was half-diluted, and their CV values remained low (between 1.53% and 3.56%), suggesting improved separation in half-diluted buffer. The run times were slightly longer in half-diluted buffer with the solvent peaks eluting later than in regular buffer (Fig. 1). The mean value of peak width at base was also higher with the half-diluted buffer (13.3 vs 11.7 min), and its CV decreased from 7.79% to 3.09%, further suggesting improved separation and reproducibility in half-diluted buffer.

Very similar average molecular weights and polydispersities were obtained for chitosans, independent of the buffer concentration (Fig. 2). The lack of influence of the acetate concentration on average M_n and M_w was confirmed by statistical analysis which yielded *p*-values from the *T*-test comparing the two buffers in the 0.129–0.951 range, with the exception of M_w from B-81-95 which increased by 5.8% in half-diluted compared to regular buffer

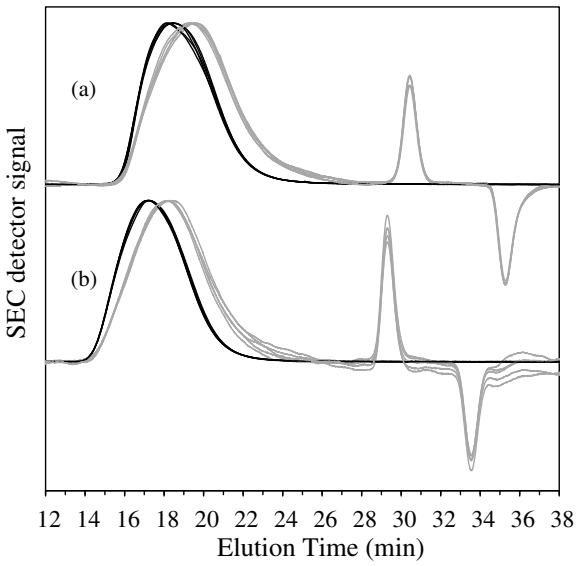

Fig. 1. SEC traces of B-92-205 on guard PW_{XL}-G6000PW_{XL}-G5000PW_{XL}: (a) mobile phase was the half-diluted buffer; (b) mobile phase was the regular buffer; black curves represent light scattering signals (at 90°), grey curves represent refractive index signals and show greater variability in (b) vs (a). Five replicates are shown for each set of conditions.

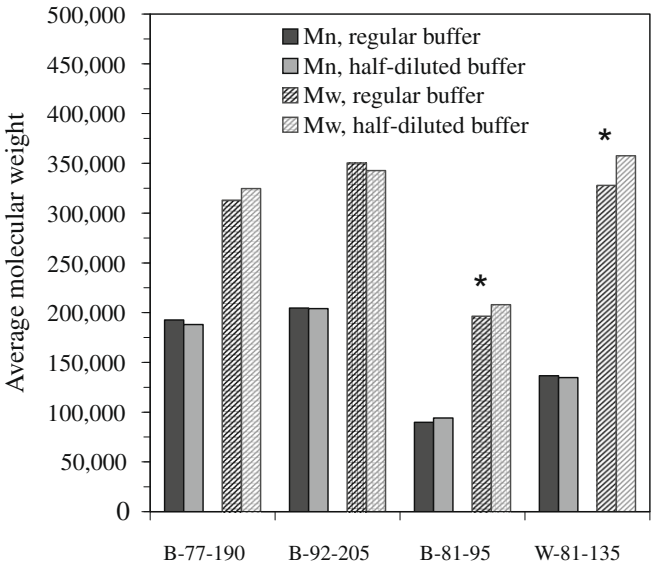

Fig. 2. Average (n = 8 or 9) molecular weights M_n, (plain-colored columns), and M_w, (striped columns), for chitosans B-77-190, B-92-205, B-81-95, and W-81-135, with replicates run on guard PW_{XL}-G6000PW_{XL}-G5000PW_{XL}, in regular buffer (black) using dn/dc = 0.198 mL/g, and in half-diluted buffer (grey) where the specifically measured dn/dc for each chitosan was used, with sample concentration at 1.00 mg/mL. The star indicates a statistically significant difference comparing regular to half-diluted buffers ($p < 0.05$ by the *T*-test).

and W-81-135 which increased by 9.1%, both of which were statistically significant ($p < 0.05$) (Table 3).

In contrast to mean values, the coefficients of variation of M_n, CV(M_n), and of M_w, CV(M_w), each calculated as standard deviation ÷ mean, were significantly reduced when determined using the half-diluted buffer compared to the regular buffer (Fig. 3). A decrease in variability of M_n was observed for all four chitosans when analyzed at lower ionic strength, with CV(M_n) decreasing from 7–12% at regular ionic strength to 3–6% at half of the regular ionic strength. This decrease in variability of M_n was statistically signif-

Table 3
Statistical analyses of M_n and M_w values of chitosans B-77-190, B-92-205, B-81-95, and W-81-135

Chitosans	*p*-value of *T*-test		*p*-variance or *p*-value in *F*-test	
	M_n	M_w	M_n	M_w
B-77-190	0.53	0.22	0.39	0.82
B-92-205	0.95	0.13	**0.0018**	**0.00076**
B-81-95	0.19	**0.0010**	**0.039**	**0.0027**
W-81-135	0.59	**0.00054**	**0.048**	0.84

p-values of the *T*-test of means, and *p*-variances (or *p*-values) of the *F*-test on variances (bold values are <0.05).

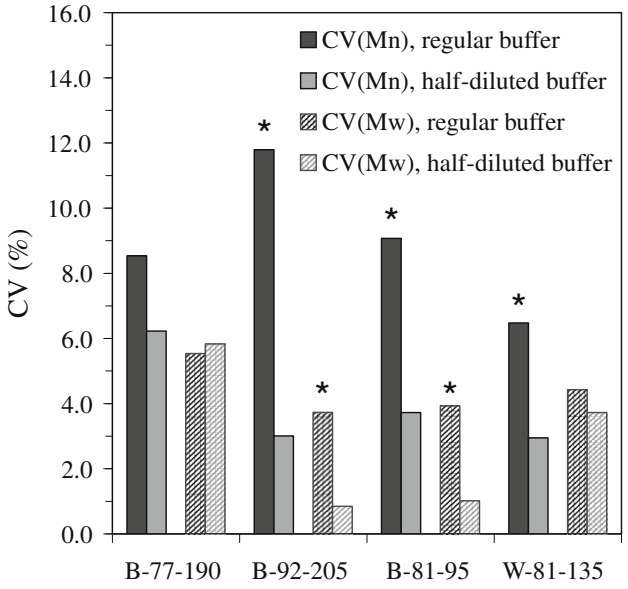

Fig. 3. Coefficients of variation of M_n, CV(M_n) (plain-colored columns), and of M_w, CV(M_w) (striped columns), for chitosans B-77-190, B-92-205, B-81-95, and W-81-135, where eight to nine replicates were run in both regular buffer (black) and half-diluted buffer (grey). The star indicates a statistically significant difference comparing regular to half-diluted buffers ($p < 0.05$ by the *F*-test). A significant reduction in variability was observed for the half-diluted buffer versus the regular buffer for 3 of 4 samples when comparing variability in M_n and for 2 of 4 samples when comparing variability in M_w.

icant for 3 of the 4 chitosans tested: B-92-205, B-81-95, and W-81-135, since the *p*-value of the *F*-test was less than 0.05 (Table 3). Similarly the CV(M_w) of two chitosans were significantly reduced from 4% to 1% (*p*-value of the *F*-test < 0.05) although two other chitosans appeared unaffected.

The important advantage of this reduced variability in chitosan SEC can be appreciated by relating viscosity changes in concentrated solutions to molecular weight changes, as we do when considering storage stability of chitosan solutions where the viscosity of concentrated chitosan solutions (2% w/v) is measured after long periods of time, up to several months. Keeping in mind that the viscosity of these concentrated solutions is proportional to the cube of M_n, a 10% underestimation of M_n, which is the typical SEC error using the regular buffer, would correspond to a 27% decrease in viscosity (0.90^3), while a 5% underestimation of M_n, which represents the error when using the half-diluted buffer, would only correspond to a 14% drop in viscosity (0.95^3). Since in many cases, solution viscosity only changes by 30% over several months at room temperature, the variability of the current SEC method using the regular buffer precludes reliable detection of changes in molecular weight associated with a 30% change in viscosity. For example one could not adequately assess whether molecular weight was being reduced due to acid hydrolysis, or whether some other process, such as a change in conformation of the chitosan chain, was responsible for declining solution viscosity. The reduced variability in SEC with the half-diluted buffer, however, now permits such hypotheses to be reliably assessed, and thereby allows greater understanding and control of molecular events responsible for altered chitosan solution properties over time. Such alterations can be very important for the function of these liquid biomaterials as tissue repair products (Hoemann et al., 2005) and as gene delivery vehicles (Lavertu et al., 2006).

4. Conclusions

In the SEC-MALLS analysis of chitosans of high molecular weight (M_n = 90–210 kDa) with TSKgel G6000PW$_{XL}$ and G5000PW$_{XL}$ columns in series, a decrease in ionic strength of the acetate buffer used in the mobile phase, from (0.3 M acetic acid and 0.2 M sodium acetate) to (0.15 M acetic acid and 0.1 M sodium acetate) did not significantly change average molecular weight values, but did significantly reduce variability of analyzed molecular weight for the four chitosans tested with a minimum of eight replicates. Coefficients of variation CV(M_n) were reduced from 7–12% in undiluted acetate buffer to 3–6% in diluted acetate buffer. This diluted buffer can therefore be recommended as mobile phase for the SEC analysis of chitosans with M_n in the range 90–210 kDa, along with analytical TSKgel PW$_{XL}$ series columns. The reduction in variability of molecular weight values resulting from SEC of chitosans represents a significant technical improvement permitting molecular weight changes to be associated with other parameters, such as solution viscosity, which are highly sensitive to the molecular weight of chitosan.

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