Carbohydrate Polymers 106 (2014) 230–237

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Determining the solution conformational entropy of oligosaccharides by SEC with on-line viscometry detection

Mallory J. Morris [a,b], André M. Striegel [a,*]

[a] Chemical Sciences Division, National Institute of Standards & Technology (NIST), 100 Bureau Drive, Mail Stop 8392, Gaithersburg, MD 20899, USA
[b] Department of Chemistry & Biochemistry, Florida State University, Tallahassee, FL 32306, USA

ARTICLE INFO

Article history:
Received 27 October 2013
Received in revised form 7 February 2014
Accepted 8 February 2014
Available online 16 February 2014

Keywords:
Size-exclusion chromatography
Viscometry
Solution conformational entropy
Oligosaccharides

ABSTRACT

Introduced here is a method for determining the solution conformational entropy of oligosaccharides ($-\Delta S$) that relies on the on-line coupling of size-exclusion chromatography (SEC), an entropically-controlled separation technique, and differential viscometry (VISC). Results from this SEC/VISC method were compared, for the same injections of the same sample dissolutions and under identical solvent/temperature conditions, to results from a benchmark SEC/differential refractometry (SEC/DRI) method which has been applied successfully over the last decade to determining $-\Delta S$ of a variety of mono-, di-, and oligosaccharides. The accuracy (as compared to SEC/DRI) and precision of SEC/VISC were found to be excellent, as was the sensitivity of the viscometer in the oligomeric region. The experiments presented here contrast three sets of $(1\rightarrow 4)$-β-D-oligosaccharides, namely manno-, cello-, and *N*-acetylchitooligosaccharides of degree of polymerization (DP) 2 through 6. For each series, the dependence of $-\Delta S$ on DP was found to be monotonic while, between series, differences at each DP could be ascribed to either the additional degrees of freedom imparted by large, multi-atomic substituent groups, or to the presence or absence of additional intramolecular hydrogen bonds, depending on the axial versus equatorial arrangement of particular hydroxyl groups. An hypothesis is advanced to explain the unexpectedly high sensitivity of viscometric detection for low-molar-mass analytes. The method presented can be extended to the analysis of oligosaccharides other than those studied here.

Published by Elsevier Ltd.

1. Introduction

In addition to their nutritive value and to their roles as the building blocks of polysaccharides, oligosaccharides are integral to a variety of biological phenomena wherein the flexibility of the sugars is key. For example, the solution flexibility of carbohydrates affects cell growth, adhesion, and apoptosis (Varki et al., 2009); the folding and routing of glycoproteins (Varki et al., 2009); and protein binding strength and signaling (Chong & Ham, 2013; Carver, 1993; Höög, Lnadersjö, & Widmalm, 2001), among others. This flexibility is directly influenced not only by solvation effects but also by the structure and conformation of the saccharides, through features that include monomer type, glycosidic linkage, anomeric configuration, degree of polymerization, and hydrogen bonding.

A direct, quantitative measure of the flexibility of carbohydrates in solution is their solution conformational entropy ΔS. Traditionally, data regarding the latter have been generated primarily, though not exclusively, by means of computer modeling employing a variety of approaches and levels of approximation (Dorsett & White, 2000; Dowd, Rockey, French, & Reilly, 2002; Schnupf, Willett, Bosma, & Momany, 2007; Schnupf, Willett, & Momany, 2010). A decade ago our group introduced size-exclusion chromatography (SEC) as a means of determining the ΔS of oligosaccharides (Striegel, 2003), by capitalizing upon the entropic nature of the separation in SEC and the fact that, to date, the SEC separation of most oligosaccharides occurs in the virtual absence of enthalpic contributions to the separation (the analysis of α- and γ-cyclodextrins under aqueous conditions being a notable exception to this (Boone, Nymeyer, & Striegel, 2008)). In these SEC analyses the chromatographic columns were connected on-line to a differential refractometer (DRI), though any other appropriate concentration-sensitive detector can be used (e.g., a UV/visible spectrophotometer could be used in the case of saccharides containing a UV-absorbing moiety, when the analytes are dissolved in a solvent with a sufficiently low UV cutoff). To date, the SEC/DRI method has been applied to the study of a variety of mono-, di-, and oligosaccharides, in both aqueous and select organic solvents, to quantitate the influence on ΔS of structural features such as anomeric configuration, degree of polymerization, linearity versus cyclicity, the $\Delta 2$ and C_3 effects, etc. (Boone et al., 2008; Boone &

* Corresponding author. Tel.: +1 301 975 3159; fax: +1 301 977 0685.
E-mail address: andre.striegel@nist.gov (A.M. Striegel).

http://dx.doi.org/10.1016/j.carbpol.2014.02.027
0144-8617/Published by Elsevier Ltd.

Striegel, 2006; Buley & Striegel, 2010; Morris & Striegel, in press; Striegel, 2003; Striegel & Boone, 2011).

Here, we introduce a new type of SEC method for the determination of ΔS, namely the coupling of SEC to a viscometric method of detection, abbreviated as SEC/viscometry or SEC/VISC. The rationale behind this approach is multifold. First, if successful, viscometric detection can provide an alternative to refractometric or other concentration-sensitive methods of detection. Second, because a viscometer can be used not only in lieu of, but also in conjunction with, a differential refractometer, the use of both detectors can serve to increase the precision of the determinations. Third, and perhaps most importantly, unlike detectors such as DRI, UV/visible, light scattering, fluorescence, infrared, etc., which are all based on spectroscopic principles, the viscometer operates based on the principles of hydrodynamics. Consequently, the viscometer should be able to provide data for samples with optical activity that may bias the results of spectroscopic techniques (e.g., absorption and/or fluorescence when trying to perform DRI measurements), or for samples which have little or no spectroscopic contrast with the solvent (a problem when trying to employ, e.g., UV/visible or infrared detection for solutions with very low molar absorptivity, or DRI or light scattering detection for solutions with very low specific refractive index increment). Fourth, while the experiments presented here employed a differential viscometer, single-capillary viscometers are among the conceptually and technologically simplest detectors available, with the potential for being built in-house (Norwood & Reed, 1997; Reed, 2005). Lastly, being able to employ differential viscometry in conjunction with a DRI (or related) detector can assist in eliminating (or confirming) an individual detector as a source of analytical problems during system troubleshooting.

The application of viscometric detection to this type of endeavor appears counterintuitive, however. The viscometer is a type of molar-mass-sensitive detector (Mourey, 2004), the response of which increases with increasing molar mass (M) of linear polymers and, vice versa, decreases as M decreases (Ekmanis, 1989). Being very low-M species, oligosaccharides would seem ill-suited to analysis by SEC with viscometric detection; many of the challenges inherent to this type of application of on-line viscometry have been described in the recent literature (Striegel, 2013a, 2013b). The present work will demonstrate, however, that analysis of oligosaccharides with degree of polymerization (DP) as low as 2 is possible by SEC/viscometry, permitting the calculation, with a high degree of precision, of the solution conformational entropy values of the members of several series of oligosaccharides. The results from the SEC/viscometry experiments show remarkable agreement with results obtained using the benchmark SEC/DRI method under identical conditions.

The three series examined here, at quasi-physiological conditions of solvent, temperature, and pH, are cello-, manno-, and *N*-acetylchitooligosaccharides. These series are coincident with one another with respect to both anomeric configuration and glycosidic linkage, these being $(1 \rightarrow 4)$-β-D in all cases. Thus, in addition to being able to determine the DP-dependence of ΔS in these series, we are also able to employ SEC/VISC to examine and quantitate how the presence of an individual axial hydroxyl group on carbon 2 influences the solution flexibility of $(1 \rightarrow 4)$-β-D-linked oligosaccharides, by comparing the manno- and cello series to each other at each DP. We also determined, by comparing to each other cello- and *N*-acetylchitooligosaccharides, the effect on ΔS of a bulky substituent on the hydroxyl groups of carbons 6 and 2′ of these $(1 \rightarrow 4)$-β-D-linked series, again as a function of DP.

The oligosaccharides examined here constitute the building blocks of cellulose, mannan, and chitin. They have found use as prebiotic candidates (cellooligosaccharides) (Akpinar, McGorrin, & Penner, 2004), and can modulate, in DP-dependent fashion, the activity of enzymes such as ribonuclease B (mannooligosaccharides) (Woods, Pathiaseril, Wormald, Edge, & Dwek, 1998). It has also been observed that the chitin synthesis activation of *Saccharomyces cerevisiae* appears specific to *N*-acetylchitoses, as said activation was not observed with cello-, isomalto-, or laminarioligosaccharides of the same DP (Becker, Piffeteau, & Thellend, 2011). Consequently, the experimental coupling of SEC and viscometry reported here provides not only a novel general method for the determination of ΔS, but also is shown to yield specific information of potential interest to the medical, biochemical, and biotechnological communities, among others.

2. Experimental

2.1. Materials

The oligosaccharides employed in this study are $(1 \rightarrow 4)$-β-D-cello-, manno, and *N*-acetylchitooligosaacharides, purchased from V-LABS, Inc. (Covington, LA) and sold to at least 95% purity by the manufacturer. The sugars were used as received, without further purification. Acetone was from J & H Berge (South Plainfield, NJ), and pullulan standard from Agilent/Varian/Polymer Laboratories (Santa Clara, CA). Hydrochloric acid and sodium azide were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Size-exclusion chromatography (SEC)

Unfiltered sample solutions (400 μL of 1 mg mL^{-1} solutions in H_2O) were analyzed with an SEC system using degassed, deionized H_2O (with 0.02% NaN_3 added to inhibit bacterial growth) as mobile phase at 1 mL min^{-1} flow rate. Eluent temperature was 37 ± 1 °C, with pH adjusted to 7.4 using 1 *M* HCl or NaOH, as needed. Separation occurred over a column bank consisting of four analytical Ultrahydrogel 6 μm particle size, nominal 120 Å pore size SEC columns, purchased from Waters Corporation (Milford, MA). Detection was performed with an Optilab rEX differential refractive index (DRI) detector and a ViscoStar differential viscometer, both from Wyatt Technology Corporation (Santa Barbara, CA). The ViscoStar has a variable-volume hold-up reservoir (see Section 2.4); in the present experiments, a hold-up volume of 27 mL was used. Columns, detectors, and injection compartment temperatures were maintained at 37 ± 1 °C. For all chromatographic determinations, results are the averages of at least six injections, three each from two separate sample dissolutions. Interdetector delays were determined, and minor flow rate fluctuations corrected, by comparing the retention time of an acetone marker peak (5 μL of acetone were added to each sample solution) in each injection, including individual cellobiose injections, to the average value of the marker peak for all cellobiose injections. Data acquisition was performed using either ASTRA software (V. 5.3.4.14) or Chromeleon (V. 6.8).

The details of viscometry and viscometric detection are given Section 2.4.

2.3. Calculation of the solution conformational entropy $-\Delta S$

Calculation of the standard conformational entropy difference between the mobile and stationary phases for the oligosaccharides in solution was based on the retention times of peak maxima (V_R), as measured by SEC, as well as the solute distribution coefficient (K_{SEC}). These two parameters are related via (Striegel, 2003, 2004; Striegel, Yau, Kirkland, & Bly, 2009)

$$K_{SEC} = \frac{V_R - V_0}{V_i - V_0} \quad (1)$$

where V_0 is the void volume of the columns and V_i is the total column volume. The internal pore volume of the system is defined as the difference between V_i and V_0. The SEC columns are quoted by

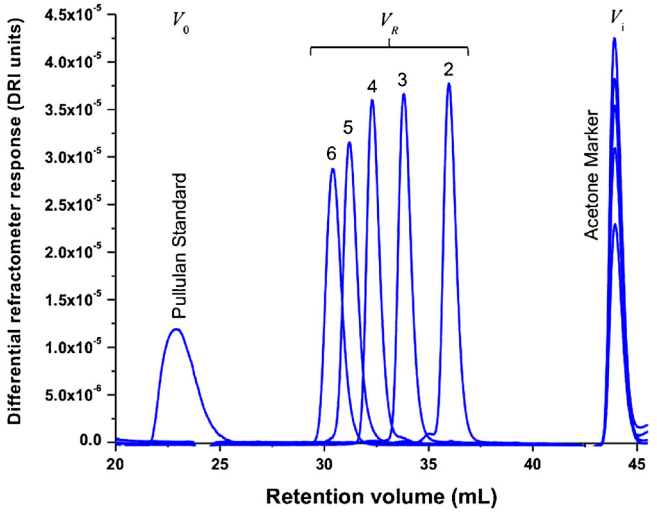


Fig. 1. SEC/DRI separation of pullulan standard, mannooligosaccharides series DP 2 through 6 (numbers above peaks), and acetone marker peak in H_2O at 37 °C and pH 7.4. K_{SEC} are determined from the retention volumes of peak maxima of the void volume V_0, the total column volume V_i, and the individual oligosaccharide samples' V_R, as per Eq. (1).

the manufacturer as having an exclusion limit of ≈5000 g mol^{-1}, based on analysis of poly(ethylene oxide) and poly(ethylene glycol) standards in water. We measured V_0 using a 22,800 g mol^{-1} narrow dispersity ($M_w/M_n \leq 1.07$) pullulan standard, and measured V_i using acetone. Fig. 1 overlays the SEC chromatogram of the mannooligosaccharides with those of the pullulan and acetone, demonstrating that the carbohydrates elute well within the separation range of the SEC columns.

When the system temperature was changed from 37 °C to 50 °C, the solute distribution coefficient K_{SEC} (shown in Table 1 for the smallest and largest members of each series) changed by only ±2% for these analytes. This strongly supports the conclusion that separation of the oligosaccharides examined here is chiefly entropic in nature (characteristic of "near-ideal" SEC behavior), as enthalpic interactions with the column packing material would lead to highly temperature-dependent values of the distribution coefficient. Consequently, we can write

$$\Delta S = R \ln K_{SEC} \tag{2}$$

Here, we have used $R = 8.3145$ J mol^{-1} K^{-1}. The standard entropy difference, $-\Delta S$, denotes the difference between the conformational entropy of the oligosaccharides in the flowing mobile phase outside the pores of the column packing versus the entropy of the oligosaccharides in the stagnant mobile phase inside the pores. The use of the negative sign (i.e., of $-\Delta S$) is a result of solute permeation in SEC being associated with a decrease in conformational entropy due to the more limited analyte mobility inside the pores as compared to analyte mobility in the interstitial volume (Striegel et al., 2009).

Table 1
Change in solute distribution coefficient K_{SEC}, as determined by SEC/DRI, as a function of temperature.

Oligosaccharide	K_{SEC} (37 °C)[a]	K_{SEC} (50 °C)[a]
Cellobiose	0.627 ± 0.001	0.617 ± 0.002
Cellohexaose	0.372 ± 0.001	0.363 ± 0.001
N,N″-Diacetylchitobiose	0.571 ± 0.001	0.565 ± 0.001
N,N″,N‴,N⁗,N⁗′-Hexaacetylchitohexaose	0.313 ± 0.001	0.309 ± 0.001
Mannobiose	0.621 ± 0.001	0.612 ± 0.001
Mannohexaose	0.357 ± 0.001	0.348 ± 0.002

[a] In H_2O at pH 7.4. Uncertainties represent one standard deviation based on at least hexuplicate determinations, three each from two different sample dissolutions (see Section 2 for details).

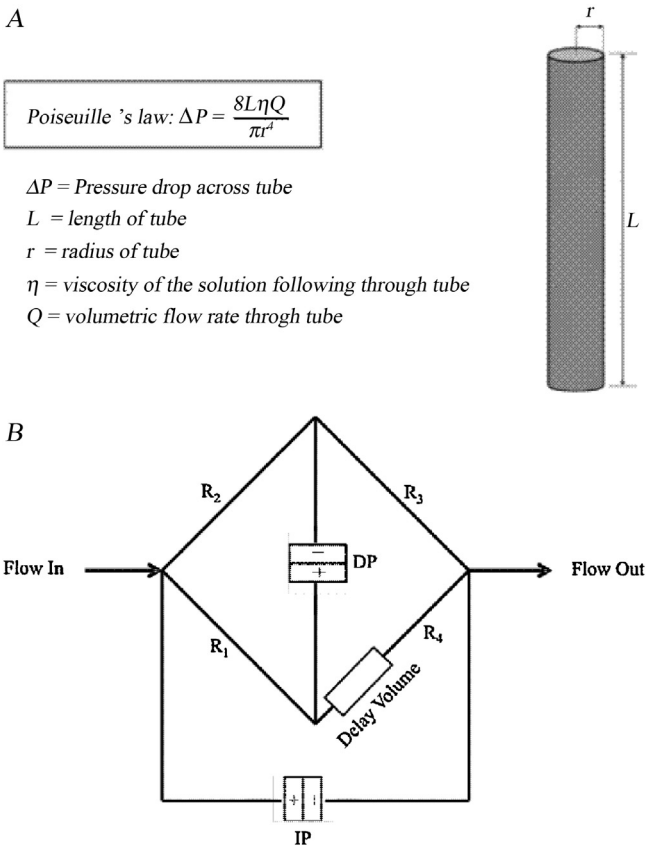


Fig. 2. (a) Principles of capillary viscometry. (b) A Wheatstone bridge-type differential viscometer, where R_1–R_4 represent individual capillaries, DP is the differential pressure transducer, and IP is the inlet pressure transducer. See text for details.

2.4. Principles of viscometric detection

The basic principle of capillary viscometry is shown in Fig. 1(a). When a constant pressure gradient drives the steady, laminar flow of solutions through a cylinder of constant cross-section, the time derivatives can be eliminated from the Navier–Stokes equations (Probstein, 1989). The simplified result is known as the Poiseuille (or Hagen–Poiseuille) equation (Haney, 1985a, 1985b; Norwood & Reed, 1997; Reed, 2005; Striegel, 2005a; Striegel et al., 2009):

$$\Delta P = \frac{8L\eta Q}{\pi r^4} \tag{3}$$

where ΔP denotes the pressure drop across the capillary; L and r and the length and inner radius of the capillary, respectively; η is the viscosity of the solution flowing through the capillary; and Q is the volumetric flow rate through the tube. Attaching a pressure transducer to the inlet and outlet of said tube provides for a single-capillary viscometer.

A more robust design than the single-capillary viscometer is that of the differential viscometer. While several types of this viscometer are available (for a discussion, see Section 9.5 of Ref. (Striegel et al., 2009)), the most popular design is that based on the Wheatstone bridge electrical circuit (Haney, 1985a, 1985b). As shown in Fig. 2(b), $R1$–$R4$ denote the four capillaries that form the bridge (with flow following Poiseuille's law in each); IP is the inlet pressure transducer, which measures the pressure drop across the bridge; DP is the differential pressure transducer (not to be confused with degree of polymerization, for which the abbreviation "DP" is also

employed here), which measures the pressure drop through the bridge; and "Delay Volume" denotes a hold-up reservoir. The ViscoStar instrument employed in the present experiments has a variable hold-up volume of 4 mL, 8 mL, 15 mL, or any combination of these volumes. Eluate flows from the column into the viscometer via the "Flow In" route and out of the viscometer into either another detector or into waste via the "Flow Out" route. For the experiments conducted in this study, the viscometer is connected in series with the differential refractometer, as described in Section 2, so that the same injections of the same dissolutions of each sample are being observed by both detectors. Disadvantages of the Wheatstone bridge design as compared to the single-capillary design are the need to determine the calibration constants of the two different types of transducers in the differential model, as these constants do not cancel each other out, and the fact that the sample is diluted by a factor of approximately two in the Wheatstone bridge detector (though, in newer models, it is possible to effect a more biased 80:20 split between the sample and solvent sides of the bridge). The latter disadvantage is compensated for by the fact that, all other factors being equal, noise in the single-capillary viscometer is several times higher than in the bridge design. Norwood and Reed found that random measurement error contributed an order of magnitude greater error to single-capillary versus bridge viscometers, affording measurements conducted with the latter a significantly higher precision than those conducted with the former (Norwood & Reed, 1997; Reed, 2005). Even when taking into account calibration error and stochastic variation from run to run, the precision of the Wheatstone bridge-type differential viscometer was found to be about twice that of its single-capillary counterpart (Norwood & Reed, 1997; Reed, 2005).

Both types of viscometer, single-capillary and differential, determine the specific viscosity η_{sp} of the solutions, defined as (Haney, 1985a, 1985b; Mourey, 2004; Norwood & Reed, 1997; Reed, 2005; Striegel, 2005a; Striegel et al., 2009):

$$\eta_{sp} \equiv \frac{\eta - \eta_0}{\eta_0} \tag{4}$$

where η and η_0 are the viscosities of the analyte (in this case, the individual oligosaccharides) solution and of the solvent, respectively. For the single-capillary viscometer, the specific viscosity of each individual chromatographic slice i, $\eta_{sp,i}$, is obtained by (Haney, 1985a, 1985b; Mourey, 2004; Norwood & Reed, 1997; Reed, 2005; Striegel, 2005a; Striegel et al., 2009):

$$\eta_{sp,i} = \frac{P_i - P_0}{P_0} = \frac{V_i - V_0}{V_0} \tag{5}$$

where P_i and V_i represent, respectively, the pressure drop along the capillary and the voltage corresponding to that pressure drop for slice i, and P_0 and V_0 are the average solvent baseline values of the same quantities.

For the Wheatstone bridge-type differential viscometer, the specific viscosity at each chromatographic slice i is determined from (Haney, 1985a, 1985b; Mourey, 2004; Norwood & Reed, 1997; Reed, 2005; Striegel, 2005a; Striegel et al., 2009):

$$\eta_{sp,i} = \frac{4DP_i}{IP_i - 2DP_i} = \frac{4K_{DP}V_{DP,i}}{K_{IP}V_{IP,i} - 2K_{DP}V_{IP,i}} \tag{6}$$

where IP and DP are the signals from the inlet and differential pressure transducers, respectively; K_{IP} and K_{DP} are the calibration constants of these transducers; and V_{IP} and V_{DP} are the voltages associated with the respective transducer responses.

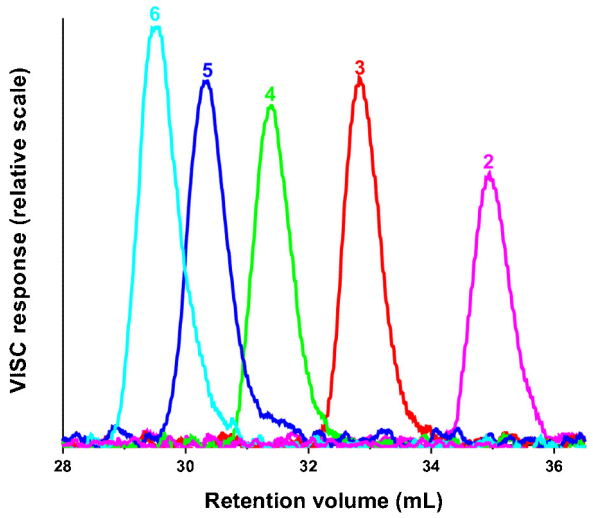


Fig. 3. Overlay of SEC/VISC chromatograms of *N*-acetylchitooligosaccharides of DP 2 through 6 (numbers above peaks), at quasi-physiological conditions. Ordinate represents relative response of the differential viscometer's differential pressure transducer. See text for details.

3. Results and discussion

3.1. SEC/VISC versus SEC/DRI

As mentioned above, the Wheatstone bridge-type differential viscometer was connected to the SEC system on-line and in series with a differential refractometer. With each detector used individually (i.e., data from both detectors were not combined, as is done, for example, when using this type of dual-detector arrangement to construct a universal calibration curve in SEC (Grubisic, Rempp, & Benoit, 1967; Striegel et al., 2009)), this SEC/VISC/DRI setup allowed the comparison of $-\Delta S$ values obtained by SEC/VISC to those derived from SEC/DRI measurements, for the exact same injections and dissolutions of each sample.

It can be observed from the overlay in Fig. 3 of the SEC/VISC chromatograms of the *N*-acetylchitooligosaccharides examined that the differential viscometer is a sufficiently sensitive detector to enable study of small sugars. Overlaid in Fig. 4 are the SEC chromatograms

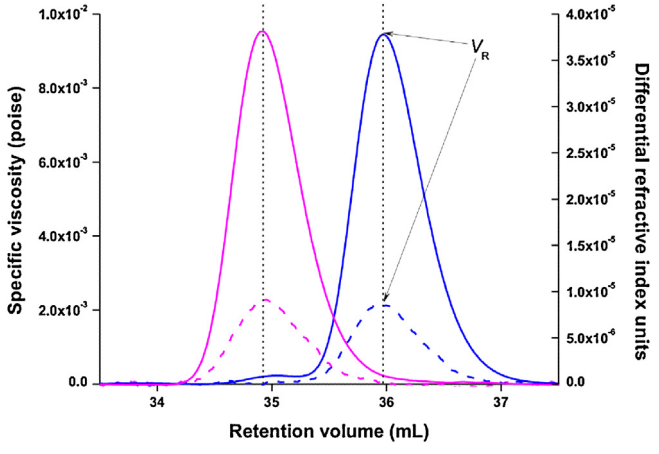


Fig. 4. SEC/VISC/DRI separation of mannobiose (blue) and *N,N′*-diacetylchitobiose (magenta) in H_2O at 37 °C and pH 7.4, as monitored by differential refractometry (DRI, solid lines) and differential viscometry (VISC, dashed lines). Vertical dotted lines are place to show the coincidence of peak maxima as determined by both detection methods subsequent to correction for interdetector delay. See text for details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Solution conformational entropy $-\Delta S$ of oligosaccharides examined, as determined by SEC/VISC.

Degree of polymerization (DP)	$-\Delta S$ (J mol^{-1} K^{-1})[a]		
	Cello	Manno	*N*-Acetylchito
2	3.881 ± 0.005	3.988 ± 0.003	4.645 ± 0.003
3	5.279 ± 0.004	5.501 ± 0.004	6.278 ± 0.006
4	6.456 ± 0.004	6.751 ± 0.004	7.577 ± 0.010
5	7.495 ± 0.002	7.786 ± 0.005	8.658 ± 0.006
6	8.403 ± 0.003	8.611 ± 0.006	9.684 ± 0.003

[a] In H_2O, at 37 °C and pH 7.4. Uncertainties represent one standard deviation based on at least hexuplicate determinations, three each from two different sample dissolutions (see Section 2 for details).

of two disaccharides, mannobiose and *N,N′*-diacetylchitobiose, as measured by both the VISC and DRI detectors. While the signal-to-noise ratio (*S*/*N*) is 4.5 times higher in SEC/DRI than in SEC/VISC, the retention times of the peak maxima (V_R) of the VISC and DRI peaks were found to differ by such small amount that K_{SEC} values determined by both methods differed from each other by less than 1 part per thousand. This was true for all oligosaccharides examined here. SEC/viscometry was thus found to possess the necessary sensitivity needed for analyzing oligosaccharides and, when compared to the benchmark SEC/DRI method of determining $-\Delta S$, the necessary accuracy as well.

3.2. Comparing the solution flexibility of cello-, manno-, and N-acetylchitooligosaccharides, as determined by SEC/VISC

The results of our experiments are given in Table 2 and Fig. 5 and demonstrate that, in addition to its accuracy and sensitivity, SEC/VISC is also a highly precise method of determining $-\Delta S$. A comparison of the three different series of oligosaccharides examined follows.

First, it can be observed that, for each series, $-\Delta S$ increases monotonically with increasing degree of polymerization (DP). This

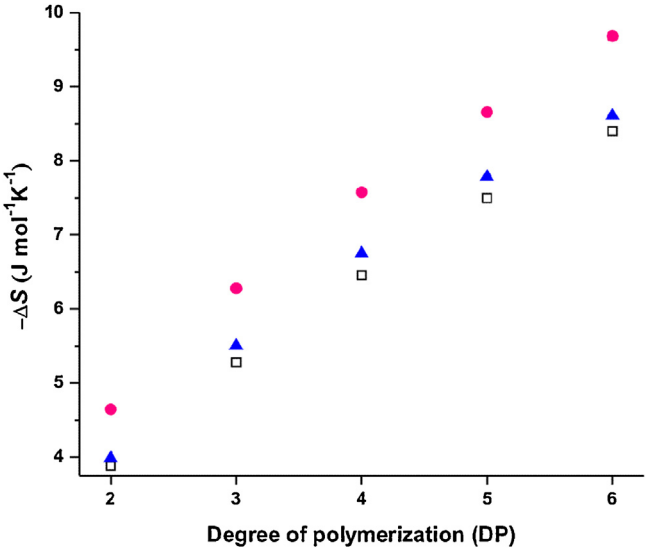


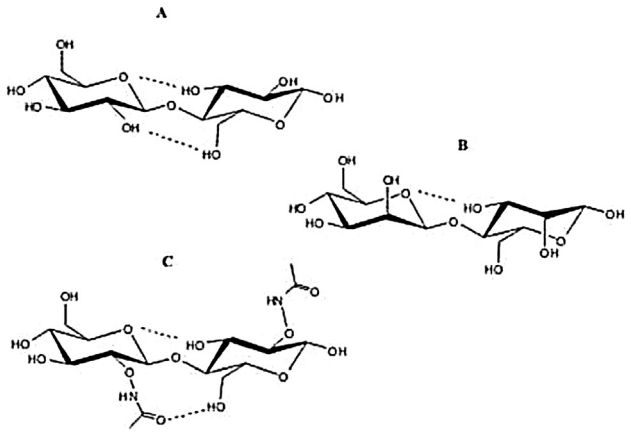
Fig. 5. Solution conformational entropy $-\Delta S$ versus degree of polymerization DP for the (1 → 4)-*β*-D-linked cellooligosaccharides (open black squares), mannooligosaccharides (filled blued triangles) and *N*-acetylchitooligosaccharides (filled magenta circles) examined. All data were obtained in H_2O at 37 °C and pH 7.4. In all cases, standard deviations (based on at least hexuplicate determinations, three each from two different sample dissolutions) are smaller than data markers and, therefore, not shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

trend is expected, as each increase in DP adds to the degrees of freedom of the molecule via an increase in the number of generalized coordinates needed to completely determine the system (Slater & Frank, 1947); all other factors being equal, larger chains are expected to be more flexible than are smaller ones. Over the DP range examined, the rate of change $\Delta|-\Delta S|/\Delta(\mathrm{DP})$ of the three series can be ranked as *N*-acetylchito > manno > cello. The differences between the $\Delta|-\Delta S|/\Delta(\mathrm{DP})$ of these series are somewhat smaller than previously measured differences between either cello- and maltooligosaccharides or between malto- and isomaltooligosaccharides under otherwise identical experimental conditions. In the case of cello- versus maltooligosaccharides, differences could be ascribed to variation in anomeric configuration between the series, as the glycosidic linkage is the same in both (Boone et al., 2008). When comparing malto- to isomaltooligosaccharides, however, it is the additional degree of freedom imparted by the methylene group in the (1 → 6) linkage that affords isomaltooligosaccharides a larger flexibility over their maltoside counterparts (Striegel & Boone, 2011).

All three series examined here are (1 → 4)-*β*-D-linked, i.e., they all possess the same anomeric configuration and glycosidic linkage. However, the additional degrees of freedom afforded by the large, multiatomic (as compared to hydrogen atoms) *N*-acetyl substituents on O_6 and O_2' of the *N*-acetylchitooligosaccharides are reflected in the substantially higher $-\Delta S$ values seen in Table 2 and Fig. 5 for members of this series, as compared to manno- and cellooligosaccharides of the same DP.

Lastly, in comparing the cello- and mannooligosaccharides to each other, the a priori expectation is for the former to be more flexible than the latter, based on the following. First, cellooligosaccharides possess an all-equatorial hydroxyl group arrangement, whereas mannooligosaccharides have one axial OH group per anhydromannose repeat unit. Extensive previous evidence has shown that, all other factors being equal, the solution flexibility of mono- and disaccharides is reduced in direct proportion to the amount of axial OH groups present (Buley & Striegel, 2010; Morris & Striegel, in press; Striegel, 2003). Second, the location of the particular axial OH of mannooligosaccharides is on carbon 2 (C_2). In the mannose repeat unit, this leads to the so-called $\Delta 2$ effect, first postulated by Reeves in 1950 (Reeves, 1950): When the axial C_2—O bond bisects the torsional angle between the two C_1—O bonds, this leads to a larger destabilization of the pyranose ring (as compared to when the C_2—O bond is equatorial), as a result of what appear to be unfavorable dipolar interactions between the C_1—O_1 and C_2—O_2 dipoles in the O_1—C_1—C_2—O_2 arrangement (Izydorczyk, 2005; Jeffrey & Yates, 1981; Juaristi & Cuevas, 1995; Kaliannan, Vishveshwara, & Rao, 1986; Lii, Chen, & Allinger, 2003; Reeves, 1950; Shallenberger, 1982; Stoddart, 1971). Extensive experiments by our group (Buley & Striegel, 2010; Morris & Striegel, in press), in both aqueous and select organic solvents, have shown that the presence of a $\Delta 2$ effect reduces the solution flexibility of aldohexoses and methyl glycosides, leading to lower values of $-\Delta S$ for those sugars with a $\Delta 2$ effect as compared to their counterparts in which this effect is absent. For example, the $-\Delta S$ of mannose, which has an axial OH on C_2 and, hence, where a $\Delta 2$ effect is present, is lower than are the $-\Delta S$ of glucose (no axial OH groups), galactose (one axial OH, on C_4), or allose (one axial OH, on C_3), at the same conditions (Buley & Striegel, 2010; Morris & Striegel, in press). Because of the axial OH group present in mannooligosaccharides but absent in cellooligosaccharides, and because of the particular location of this OH group, the $-\Delta S$ of mannooligosaccharides was expected to be lower than that of cellooligosaccharides examined under the same experimental conditions.

The experimental results, however, differ from the above expectations. As seen in Table 2 and Fig. 5, at each DP the solution conformational entropies of the mannoligosaccharides examined



A = Cellobiose; **B** = Mannobiose; **C** = *N,N′*-Diacetylchitobiose
(Dashed lines indicate intramolecular hydrogen bonding)

Scheme 1.

exceed those of the respective cellooligosaccharides. As shown in Scheme 1, intramolecular hydrogen bonding occurs between the hydrogen of the OH group on carbon three of one pyranose ring and the ring oxygen of the contiguous pyranose unit of all three (1 → 4)-*β*-D-linked oligosaccharides in this study, a bonding denoted here as $HO_3\cdots O_5'$. Cellooligosaccharides, however, possess an additional intramolecular hydrogen bond, between the oxygen of the OH group on carbon 6 of one ring and the hydrogen of the equatorial OH group on the next ring ($O_6\cdots HO_2'$). In mannooligosaccharides, the hydroxyl group on C_2 is axially oriented (which gives rise to the aforementioned Δ2 effect in mannose), preventing this second H-bond from forming (Mackie, Sheldrick, Akrigg, & Perez, 1986). The additional H-bond in the cellooligosaccharides contributes to maintaining these sugars in a more rigid conformation; consequently, their flexibility in solution is lower than that of the mannooligosaccharides (Moreira & Filho, 2008), a fact reflected in the relative $-\Delta S$ values of these series.

Like the cellooligosaccharides, the *N*-acetylchitooligosaccharides also have a second intramolecular H-bond, in the latter case between the hydrogen of HO_6 and the carbonyl oxygen of the acetamide group on C_2 of the contiguous ring. Because of the additional distance provided by the —NH—C(CH_3)═O group (Scheme 1), however, this second H-bonding interaction in the *N*-acetylchito series is not as strong as is it is in the cello series. The additional degrees of freedom imparted to the molecules by the acetamide substituent groups, discussed above, are sufficient to afford the *N*-acetylchito series the largest $-\Delta S$, at each DP, of the series examined, in spite of the presence of two intramolecular H-bonds.

3.3. Regarding the applicability of viscometric detection to oligosaccharide analysis

We address here the suitability of viscometric detection to the study of oligosaccharides. As mentioned in the Introduction, there are several reasons why viscometry is not expected to possess the necessary sensitivity for studying dilute oligomer solutions, most notably the fact that the sensitivity of viscosity detectors decreases with decreasing molar mass and oligosaccharides are, on a polymeric scale, very low molar mass analytes. As demonstrated above, however, the *S*/*N* of viscometric traces of all oligosaccharides examined was quite good, and the precision of the determinations was excellent. The following hypothesis aims to explain these results.

It is well known that, from a light scattering standpoint, oligomers are considered highly anisotropic objects (Kojo, Osa, Yoshizaki, & Yamakawa, 2003; Striegel, 2005b). Evidence for this comes from the ability of oligomers to depolarize scattered light much more so than their polymeric counterparts (Striegel, 2005b). By light scattering standards, while most (though certainly not all) macromolecules generally behave as random coils, the interaction of most oligomers with the electric vector of the incident radiation is rodlike, an interaction that can result in the depolarization of scattered light.

The interaction of oligomers with a hydrodynamic flow field appears to be qualitatively different than their interaction with incident radiation. To better understand this behavior, let us begin with the definition of intrinsic viscosity [*η*] and with the latter's relation to molar mass *M* via the well-known Mark–Houwink relationship (Haney, 1985a, 1985b; Mourey, 2004; Norwood & Reed, 1997; Reed, 2005; Striegel, 2005a; Striegel et al., 2009):

$$[\eta] \equiv \lim_{c\to 0} \frac{\eta_{sp}}{c} = KM^a \tag{7}$$

Here, *K* and *a* are constants that depend on polymer chemistry and architecture as well as on dilute solution thermodynamics, *c* is the concentration of analyte in a near-infinitely dilute solution, and η_{sp} is the specific viscosity of the solution, defined as per Eq. (4). Several theoretical values for the exponent *a* are known (Striegel, 2005a; Striegel et al., 2009), in particular $a = 0.5$ for linear random coils at theta conditions, $a \approx 0.6$–1.0 for linear random coils at thermodynamically good solvent/temperature conditions, $a = 2$ for rigid rods, etc. For homogeneous hard spheres (hard spheres of constant density and composition), $a = 0$. Experimental support for the latter value can be found in multi-detector SEC studies of highly branched polymers, including stars with high branching functionality (Podzimek, 2011), and in studies of latex suspensions using hydrodynamic chromatography coupled to DRI, VISC, and multi-angle static light scattering (Brewer & Striegel, 2010; Striegel, 2011). In these experiments, Mark–Houwink plots of [*η*] versus *M*, which each axis plotted on a logarithmic scale, found [*η*] to be essentially invariant to changes in *M* even, in the case of latexes, over several orders of magnitude in molar mass (in a Mark–Houwink plot, the slope is equal to the exponent *a* in Eq. (7), so that [*η*] remaining constant while *M* varies results in a value of $a = 0$).

As can be seen from Fig. 6, within each of the three series examined here the response of the viscometer shows little variation with DP. The variation that is observed is substantially smaller than would be expected if the oligosaccharides behaved as linear random coils and more akin to the variation (or lack thereof) displayed by hard spheres, especially for the case the manno- and cellooligosaccharides. The response of the viscometer (or, more specifically, that of the differential viscometer's differential pressure transducer which, as discussed above, measures the pressure drop through the bridge) is proportional to the specific viscosity of the solutions. If said specific viscosity is invariant to DP (i.e., to *M*) over the oligomeric range examined, this corresponds to the case of $a = 0$ and then the viscometer becomes a *de facto* concentration-sensitive detector, as a result of:

$$[\eta] = \lim_{c\to 0} \frac{constant}{c} = K, \text{ when } a = 0 \tag{8}$$

where the value of the *constant* in the numerator depends on the particular set of analytes (particular oligosaccharide series).

This type of behavior (constancy in viscometric response with increasing degree of polymerization) matches that of the oligosaccharides examined. The only series to show some viscometric dependence on DP is that of the *N*-acetylchitooligosaccharides, which is not surprising given that these are the most flexible of

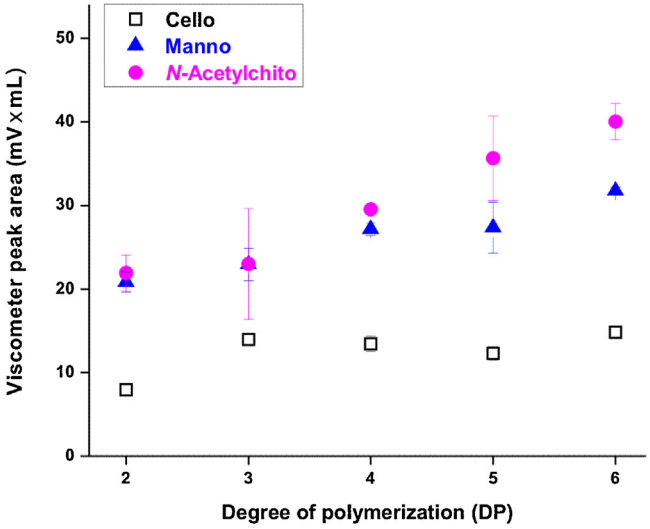


Fig. 6. Areas of SEC/VISC peaks (as monitored by the differential pressure transducer of the viscometer) as a function of degree of polymerization of cellooligosaccharides (open black squares), mannooligosaccharides (solid blue triangles), and *N*-acetylchitooligosaccharides (filled magenta circles). All data were obtained in H_2O at 37 °C and pH 7.4. Standard deviations are based on at least hexuplicate determinations, three each from two different sample dissolutions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

all the analytes examined, i.e., those which, viscometrically, most resemble linear random coils rather than hard spheres.

4. Conclusions

We demonstrate here how SEC/viscometry can be employed to determine the solution conformational entropy of oligosaccharides. By contrasting results to those obtained by SEC/DRI analysis of the same injections of the same sample dissolutions, under the same experimental condition, SEC/VISC was found to compare well to the benchmark SEC/DRI method of determining $-\Delta S$ of small sugars, displaying excellent accuracy and precision and good sensitivity. While the latter is not as high (in the present cases) as is the sensitivity of refractometric detection, there are a number of potential advantages to the use of viscometric detection for the purposes employed here, notably when/if applied to samples with undesirable spectroscopic properties such as strong absorption or fluorescence, or in cases where the analyte solutions display negligible optical contrast with the solvent.

In the SEC/VISC experiments used to introduce this technique for the determination of the solution conformational entropy of oligosaccharides, three series of $(1\rightarrow4)$-β-D-linked sugars, in each case covering degrees of polymerization 2 through 6, were examined and compared to each other. For each series, $-\Delta S$ was observed to increase monotonically with DP. Of the three series, *N*-acetylchitooligosaccharides were observed to have a higher flexibility in solution (larger $-\Delta S$ values) at each DP, due to the additional degrees of freedom provided the molecules by the *N*-acetyl substituents. While a priori expectation (based on the $\Delta2$ effect in mannopyranose, an effect which is absent in glucopyranose) was for the cellooligosaccharides to display higher flexibility than their manno- counterparts, the opposite was observed. An explanation for this behavior appears to lie in the way the axial OH on C_2 of the mannooligosaccharides prevents the formation of a second intramolecular H-bond in these sugars. This second H-bond is favored by the all-equatorial OH arrangement in the cello-series and serves to hold these in a more rigid solution conformation than mannosaccharides of the same DP.

Because its response is directly proportional to molar mass, viscometry was not expected to possess the necessary sensitivity to probe the oligomeric region of carbohydrates. However, the differential viscometer employed in the present experiments produced satisfactory response at all DP, even in the case of disaccharides, and should work for monosaccharides, as well. It was hypothesized that, while oligomers may be considered highly anisotropic objects from a light scattering point-of-view, from a hydrodynamic standpoint they may be considered as near-spherical objects for which the viscometer's response is nearly independent of molar mass, with the detector instead becoming a type of concentration-sensitive detector in this low-*M* region.

The experiments and results presented here should be of interest to the carbohydrate and modeling communities, as well as to analytical and polymer scientists and to those performing research in the areas of glycovaccines, nutrition, and food science, among others.

Acknowledgements

The authors would like to thank V-LABS, Inc. and, in particular, Sharon Vercellotti for help in securing many of the needed analytes. Commercial products are identified to specify adequately the experimental procedure. Such identification does not imply endorsement or recommendation by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

References

Akpinar, O., McGorrin, R. J., & Penner, M. H. (2004). Cellulose-based chromatography for cellooligosaccharide production. *Journal of Agricultural and Food Chemistry*, *52*, 4144–4148.

Becker, H. F., Piffeteau, A., & Thellend, A. (2011). *Saccharomyces cerevisiae* chitin biosynthesis activation by N-acetylchitooses depends on size and structure of chito-oligosaccharides. *BMC Research Notes*, *4*, 454–459.

Boone, M. A., Nymeyer, H., & Striegel, A. M. (2008). Determining the solution conformational entropy of O-linked oligosaccharides at quasi-physiological conditions: Size-exclusion chromatography and molecular dynamics. *Carbohydrate Research*, *343*, 132–138.

Boone, M. A., & Striegel, A. M. (2006). Influence of anomeric configuration, degree of polymerization, hydrogen bonding, and linearity versus cyclicity on the solution conformational entropy of oligosaccharides. *Macromolecules*, *39*, 4128–4131.

Brewer, A. K., & Striegel, A. M. (2010). Hydrodynamic chromatography of latex blends. *Journal of Separation Science*, *33*, 3555–3563.

Buley, T. D., & Striegel, A. M. (2010). Relation between the $\Delta2$ effect and the solution conformational entropy of aldohexoses and select methyl glycosides. *Carbohydrate Polymers*, *79*, 241–249.

Carver, J. P. (1993). Oligosaccharides: How can flexible molecules act as signals? *Pure and Applied Chemistry*, *65*, 763–770.

Chong, S. H., & Ham, S. (2013). Conformational entropy of an intrinsically disordered protein. *Journal of Physical Chemistry B*, *117*, 5503–5509.

Dorsett, H., & White, A. (2000). *Overview of molecular modelling and ab initio molecular orbital methods suitable for use with energetic materials. DSTO-GD-0253*. Salisbury, Australia: DSTO Aeronautical and Maritime Research Laboratory.

Dowd, M. K., Rockey, W. M., French, A. D., & Reilly, P. J. (2002). MM3 modeling of aldopentose pyranose rings. *Journal of Carbohydrate Chemistry*, *21*, 11–25.

Ekmanis, J. L. (1989). GPC analysis of polymers with an on-line viscometer detector. In *1989 international GPC symposium proceedings* (pp. 1–25). Milford, MA: Waters Corporation.

Grubisic, Z., Rempp, P., & Benoit, H. (1967). A universal calibration for gel permeation chromatography. *Journal of Polymer Science: Polymer Letters*, *5*, 753–759.

Haney, M. A. (1985a). The differential viscometer. I. A new approach to the measurement of specific viscosities of polymer solutions. *Journal of Applied Polymer Science*, *30*, 3023–3036.

Haney, M. A. (1985b). The differential viscometer. II. On-line viscosity detector for size-exclusion chromatography. *Journal of Applied Polymer Science*, *30*, 3037–3049.

Höög, C., Lnadersjö, C., & Widmalm, G. (2001). Oligosaccharides display both rigidity and high flexibility in water as determined by ^{13}CNMR relaxation and ^{1}H,^{1}H NOE spectroscopy: Evidence of *anti-*ϕ and *anti-*ψ torsions in the same glycosidic linkage. *Chemistry – A European Journal*, *7*, 3069–3077.

Izydorczyk, M. (2005). Understanding the chemistry of food carbohydrates. In S. W. Cui (Ed.), *Food carbohydrates – Chemistry, physical properties, and applications* (pp. 1–65). Boca Raton, FL: Taylor & Francis.

Jeffrey, G. A., & Yates, J. H. (1981). Application of *ab initio* molecular-orbital calculations to the structural moieties of carbohydrates. *Carbohydrate Research*, *96*, 205–213.
Juaristi, E., & Cuevas, G. (1995). *The anomeric effect*. Boca Raton, FL: CRC Press.
Kaliannan, P., Vishveshwara, S., & Rao, V. S. R. (1986). Anomeric effect in carbohydrates – An ab initio study on extended model systems. *Proceeding of the Indian Academy of Science (Chemical Science)*, *96*, 327–339.
Kojo, H., Osa, M., Yoshizaki, T., & Yamakawa, H. (2003). Mean-square optical anisotropy of oligo- and poly(α-methylstyrene)s in dilute solution. *Macromolecules*, *36*, 6570–6576.
Lii, J.-H., Chen, K.-H., & Allinger, N. L. (2003). Alcohols, ethers, carbohydrates, and related compounds. IV. Carbohydrates. *Journal of Computational Chemistry*, *24*, 1504–1513.
Mackie, W., Sheldrick, B., Akrigg, D., & Perez, S. (1986). Crystal and molecular structure of mannotriose and its relationship to the conformations and packing of mannan and glucomannan chains and mannobiose. *International Journal of Biological Macromolecules*, *8*, 43–51.
Moreira, L. R. S., & Filho, E. X. F. (2008). An overview of mannan structure and mannan-degrading enzyme systems. *Applied Microbiology and Biotechnology*, *79*, 165–178.
Morris, M. J., & Striegel, A. M. (2014). Does the "C3 effect" offset the Δ2 effect, as regards the solution flexibility of aldoses? *Biopolymers*, http://dx.doi.org/10.1002/bip.22446 (in press)
Mourey, T. H. (2004). SEC molecular-weight-sensitive detection. *International Journal of Polymer Analysis and Characterization*, *9*, 97–135.
Norwood, D. P., & Reed, W. F. (1997). Comparison of on-line single-capillary and bridge capillary viscometric detectors for size exclusion chromatography. *International Journal of Polymer Analysis and Characterization*, *4*, 99–132.
Podzimek, S. (2011). *Light scattering, size exclusion chromatography and asymmetric flow field flow fractionation*. New York: Wiley.
Probstein, R. F. (1989). *Physicochemical hydrodynamics*. Boston: Butterworths.
Reed, W. F. (2005). Fundamentals of static light scattering and viscometry in size-exclusion chromatography and related methods. In A. M. Striegel (Ed.), *Multiple detection in size-exclusion chromatography; ACS symposium series* (Vol. 893) (pp. 13–51). Washington, DC: American Chemical Society.
Reeves, R. E. (1950). The shape of pyranoside rings. *Journal of the American Chemical Society*, *72*, 1499–1506.
Schnupf, U., Willett, J. L., Bosma, W. B., & Momany, F. A. (2007). DFT study of α- and β-D-allopyranose at the B3LYP/6-311++G** level of theory. *Carbohydrate Research*, *342*, 196–216.
Schnupf, U., Willett, J. L., & Momany, F. (2010). DFTMD studies of glucose and epimers: Anomeric ratios, rotamer populations, and hydration energies. *Carbohydrate Research*, *345*, 503–511.
Shallenberger, R. S. (1982). *Advanced sugar chemistry*. Westport, CT: AVI.
Slater, J. C., & Frank, N. H. (1947). *Mechanics*. New York: McGraw-Hill.
Stoddart, J. F. (1971). *Stereochemistry of carbohydrates*. New York: Wiley.
Striegel, A. M. (2003). Anomeric configuration, glycosidic linkage, and the solution conformational entropy of O-linked disaccharides. *Journal of the American Chemical Society*, *125*, 4146–4148 (see erratum in Striegel, A. M. (2004). *Journal of the American Chemical Society*, *126*, 4740).
Striegel, A. M. (2004). Thermodynamic equilibrium of the solute distribution in size-exclusion chromatography. *Journal of Chromatography A*, *1033*, 241–245.
Striegel, A. M. (2005a). Multiple detection in size-exclusion chromatography of macromolecules. *Analytical Chemistry*, *77*, 104A–113A.
Striegel, A. M. (2005b). Depolarized multiangle light scattering coupled with size-exclusion chromatography: Principles and select applications. In A. M. Striegel (Ed.), *Multiple detection in size-exclusion chromatography; ACS symposium series* (Vol. 893) (pp. 76–93). Washington, DC: American Chemical Society.
Striegel, A. M. (2011). 7 faces of Dr. Lao: The many roles of light scattering in multi-detector polymer separations. In *22nd international light scattering symposium proceedings* (pp. 6/1–6/31). Santa Barbara, CA: Wyatt Technology Corporation.
Striegel, A. M. (2013a). Size-exclusion chromatography of asphaltenes: An experimental comparison of commonly used approaches. *Chromatographia*, *76*, 725–733.
Striegel, A. M. (2013b). There's plenty of gloom at the bottom: The many challenges of accurate quantitation in size-based oligomeric separations. *Analytical and Bioanalytical Chemistry*, *405*, 8959–8967.
Striegel, A. M., & Boone, M. A. (2011). Influence of glycosidic linkage on solution conformational entropy of oligosaccharides: Malto- vs. isomalto and cello- vs. laminarioligosaccharides. *Biopolymers*, *95*, 228–233.
Striegel, A. M., Yau, W. W., Kirkland, J. J., & Bly, D. D. (2009). *Modern size-exclusion liquid chromatography* (2nd ed.). New York: Wiley.
Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., et al. (2009). *Essentials of glycobiology* (2nd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Woods, R. J., Pathiaseril, A., Wormald, M. R., Edge, C. J., & Dwek, R. A. (1998). The high degree of internal flexibility observed for an oligomannose oligosaccharide does not alter the overall topology of the molecule. *European Journal of Biochemistry*, *258*, 372–386.