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Cellulose-Based Chromatography for Cellooligosaccharide Production

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The potential of using cellulose stationary phases for the chromatographic fractionation of cellooligosaccharide preparations has been explored. The impetus for the work is the current interest in using cellooligosaccharides as functional nondigestible oligosaccharides in foods. The conceptual studies illustrate the potential of using ethanol−water mobile phases in conjunction with cellulose stationary phases for cellooligosaccharide fractionation. Cellooligosaccharide solubility in ethanol−water mixtures and their elution order from cellulose-based columns using ethanol−water mobile phases were shown to be in line with their degree of polymerization (DP), with the higher DP cellooligosaccharides being less soluble and having longer retention times. The retention volume for all COS increased with increased temperature. Both microcrystalline and fibrous cellulose preparations were shown to work as chromatographic stationary phases. The application experiments demonstrate the potential of using cellulose stationary phases for the cleanup and fractionation of cellooligosaccharide mixtures generated via acid-catalyzed hydrolysis of cellulose.

KEYWORDS: Cellooligosaccharides; cellulose; chromatography; fractionation

INTRODUCTION

Functional foods, commonly defined as those "... demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" (*1*), are important from both health and economic perspectives. Within the general class of functional foods, prebiotics are receiving considerable attention (*2*−*4*). Prebiotics were originally defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (*5*). Nondigestible oligosaccharides (NDO), which are resistant to digestion by human gastric and pancreatic enzymes, are likely prebiotic candidates (*6*). The impact of NDOs on bowel physiology is largely a result of their fermentation within the large intestine. NDO preparations can be prepared in several ways, including extractions, chemical and enzymatic condensations, and/or controlled hydrolysis of parent polysaccharides (*7*). A number of such preparations have been investigated (*8*).

Cellooligosaccharides (COS) are potentially the most widely available of the NDOs, in that the parent polysaccharide is the largest carbon sink in the biosphere. These linear oligosaccharides are composed of 1,4-linked β-D-glucopyranose moieties. Studies with humans and rodents have suggested beneficial effects related to carbohydrate metabolism, diabetes and obesity associated with COS intake (*9*, *10*). Their effect on the microbial ecosystem of the colon has yet to be determined. The application of COS as NDOs in foods will require practical routes for the preparation of COS products with defined chemical/physical properties − as is the case with the NDOs currently used in the food industry (*11*). The optimum situation would be to develop COS/NDO preparations covering a range of functional properties, similar to what is currently available with maltodextrin products (*12*).

COS are typically prepared by acid-catalyzed hydrolysis of cellulose, followed by fractionation/purification of the resulting liquid phase. Fractionation schemes have included size-exclusion, ion-mediated, and adsorption chromatography (*13*, and references therein). Size exclusion, using polyacrylamide gel stationary phases and water as the mobile phase, and adsorption, using charcoal/Celite stationary phases and water/ethanol mobile phases, are most commonly used for preparative purposes (*13*). In this study, we explored the potential of using cellulose as the chromatographic stationary phase for COS cleanup. The rationale is to exploit the affinity of COS for its parent polysaccharide, cellulose. Cellulose itself is a relatively inexpensive stationary phase, and it would be readily available at COS processing facilities. The results demonstrate that cellulose stationary phases, combined with water-ethanol mobile phases, can be used to obtain COS preparations with a unique degree of polymerization profiles, and hence, functional properties.

MATERIALS AND METHODS

Materials. The Aminex HPX-42A column was purchased from Bio-Rad Laboratories, Hercules, California; fibrous cellulose powder (CF 11) and LK5D (150 Å) TLC plates from Whatman Chemical Division,

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Clifton, New Jersey; microcrystalline cellulose (Avicel PH 105) from FMC Corp., Rockland, ME; *p*-anisaldehyde from Aldrich Chemical Co., Milwaukee, WI; 4,4′ dicarboxy-2,2′-biquinoline (disodium salt of bicinchonic acid), glucose and cellobiose from Sigma Chemical Co., St. Louis, MO. Cellooligosaccharide standards cellotriose (G3) through cellohexaose (G6) were obtained using a charcoal−Celite column chromatography method based on that of Miller et al. (*14*) after controlled acid hydrolysis of cellulose. The fractions corresponding to each cellooligosaccharide were combined and freeze-dried.

Preparation of Water Soluble Cellooligosaccharides. Soluble cellooligosaccharides were prepared according to Miller et al. (*14*), with slight modification. A 10-g sample of fibrous cellulose powder was dissolved in 100 mL of concentrated HCl, pre-cooled to −30 °C, and stirred for 15−20 min. The temperature was then raised to 25 °C and the stirring continued for 2−3 h. The solution was then slowly added to 725 mL 4 °C 1-propanol and stirred for 15 min. The resulting precipitate was collected after centrifugation at 5000*g* for 5 min. The pellet was washed with technical grade ethanol until the pH reached 5−6. Finally, the pellet was extracted with 400 mL of cold double-distilled water. The insoluble material was removed by centrifugation and decantation. The cellooligosaccharides in the aqueous phase were concentrated by rotary evaporation under reduced pressure and freeze-dried (yielding approximately 300 mg). The DP profile of the resulting cellooligosaccharide mixture was determined by HPLC analysis, as described below.

Ethanol and Cellulose Effect on Cellooligosaccharide Solubility. A 1-mg sample of COS preparation was added to 1 mL of 0, 20, 40, 60, 80, or 90% ethanol-in-water solutions. These test solutions were mixed thoroughly and allowed to stand at ambient (∼22 °C) temperature for 1 h. The resulting solutions/suspensions were centrifuged at 5000*g* for 5 min, and the DP profile of the soluble phase was determined by HPLC as described below. The effect of microcrystalline cellulose on COS solubility was investigated by adding 9 mg of the appropriate cellulose with 1 mg of COS preparation to 1 mL of 0, 20, 40 and 60% ethanol-in-water solutions. The resulting suspensions were mixed and allowed to stand at ambient temperature for 2 h. The resulting suspensions were processed and analyzed as described for those test solutions not containing cellulose.

Cellooligosaccharide Chromatography with Cellulose Stationary Phases. Fibrous and microcrystalline cellulose were tested as chromatographic media in conjunction with water/ethanol mobile phases. Cellulose preparations were repeatedly washed with water at room temperature to remove impurities and fines prior to their being packed, using gravity feed, into 2- × 25-cm columns. The columns were then equilibrated with the starting mobile phase (starting mobile phases differed depending on the experiment, see Results and Discussion) by use of a peristaltic pump. A representative COS preparation, ∼100 mg, was dissolved in 10 mL of the same mobile phase and then introduced onto the column. The flow rate was 0.1 mL/min, and fraction volume was 1 mL. The column eluate was monitored for the presence of COS, as reducing sugars, using a 2,2′-bicinchoninate-based assay (*15*). Fractions showing the presence of reducing sugars were further analyzed by thin layer chromatography (silica plates, ethyl acetate/methanol/water, 40:20:15, mobile phase, *p*-anisaldehyde-sulfuric acid visualizing reagent) to determine the DP of the COS therein (*16*). On the basis of the TLC results, fractions containing homologous COS were pooled and COS quantified via HPLC analyses, as described below. Following each chromatographic run, columns could be regenerated by washing with water at room temperature until no further reducing sugars could be detected.

Application Studies. *Experiment 1.* Soluble COS were prepared as described above with the exception that the COS and cellulose-containing pellet obtained following centrifugation of the 1-propanol-diluted hydrolysis mixture was suspended in 100% ethanol. This suspension was packed into an empty 2.5- × 45-cm column, thus providing a uniform mixture of cellulose stationary phase and associated COS. In this experiment, the cellulose stationary phase was entirely comprised of the cellulose used in the initial hydrolysis step. The column was then washed with ethanol until the apparent pH of the eluent was ∼6. Soluble cellooligosaccharides were eluted using a linear ethanol (200 mL) → water (200 mL) gradient. Flow rate was 0.1 mL/min, and fraction volume was 1 mL. The eluate was monitored via reducing sugar assays, TLC, and HPLC, as described below.

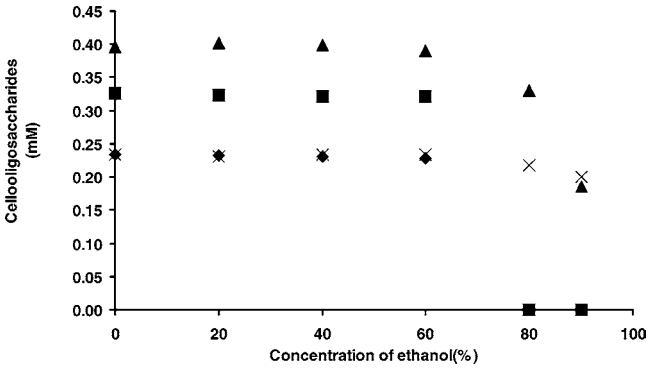

Figure 1. Effect of ethanol on cellooligosaccharide solubility. Test solutions were prepared by adding 1 mg of mixed cellooligosaccharide preparation per milliliter of solvent. Replicate measurements were within 7%. (cellotriose, ×; cellotetraose, ▲; cellopentaose, ■; cellohexaose, ◆).

Experiment 2. Soluble COS were prepared as described above, with the exception that following the 2−3 h reaction period at 25 °C, the solution was poured into 400 mL of ice-cold water and subsequently neutralized, to ∼pH 6, with solid $NaHCO_3$. Solids were removed via centrifugation (5000*g* for 5 min), and the supernatant was made 60% in ethanol. Any additional precipitate was removed prior to beginning chromatography. A 200-mL aliquot of the neutralized COS in 60% ethanol solution was then loaded on a 60% ethanol-equilibrated 5- × 25-cm column packed with fibrous cellulose. The column was run as a one step gradient, using 60% ethanol as the initial eluent (for elution of lower DP COS and salts) and water as the final eluent. Flow rate was 0.5 mL/min, and fraction volume was 7.5 mL. Column eluate was monitored for the presence of COS, as reducing sugars, using a 2,2′-bicinchoninate-based assay.The eluate was monitored for reducing sugars by TLC and by HPLC, as described below.

HPLC Analyses of Cellooligosaccharides. Cellooligosaccharide mixtures dissolved in water, the sample from solubility study, and column fractions were chromatographed on a Water's HPLC system equipped with a differential refractometer (Model 410), autosampler (Model 717) and column oven. Before injection, samples were filtered through a 0.45-μm filter. Aliquots of filtered sample (50 μL) were injected to the HPLC system. COS were eluted using distilled−deionized water as the mobile phase from an ion-mediated stationary phase in the silver form (Aminex HPX-42A, Bio-Rad Inc.) (*17*). The column (300- × 7.8-mm), which was preceded by its complimentary de-ashing cartridge (Bio-Rad), was used at 85 °C and a flow rate of 0.4 mL per minute. A completed analysis of cellooligosaccharide was carried out for less than 30 min. After 30 min, because some samples were injected in ethanol solution, an ethanol peak was eluted. Sample running time was 60 min, 30 min for data collection, and 30 min for postrun. A computing integrator determined the start, retention time, and end of the peak, and integrated the area under each peak as a function of height and width of the peak. Concentration of oligosaccharide was quantified using average peak areas compared with mixture of standard oligosaccharide (G1 through G6) and expressed as mM oligosaccharide.

RESULTS AND DISCUSSIONS

Initial experiments focused on the extent to which ethanol, a relatively nontoxic and inexpensive solvent, affects COS solubility. Experiments were done using a representative COS mixture containing, on a mole-fraction basis, 0.23 cellotriose (G3), 0.40 cellotetraose (G4), 0.33 cellopentaose (G5), and 0.23 cellohexaose (G6) − with negligible amounts of glucose (G1), cellobiose (G2), and cellooligosaccharides of DP > 6. **Figure 1** illustrates the large decline in cellotetraose, cellopentaose, and cellohexaose solubility as ethanol concentrations were increased

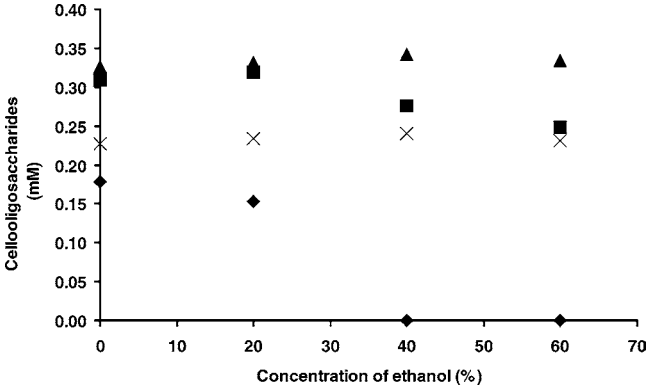

Figure 2. Effect of ethanol on cellooligosaccharide solubility in the presence of cellulose. Test solutions were prepared by adding 1 mg of mixed cellooligosaccharide preparation and 9 mg of microcrystalline cellulose per milliliter of solvent. Replicate measurements were within 7%. (cellotriose, ×; cellotetraose, ▲; cellopentaose, ■; cellohexaose, ◆).

above 60%; cellopentaose and cellohexaose being essentially insoluble at ethanol concentrations ≥80%. The results demonstrate that moderate-to-high ethanol concentrations profoundly influence COS solubility, the extent of the effect being dependent on the degree of polymerization of the COS. This lends credence to ethanol-based cleanup protocols applied to heterogeneous COS preparations (*18*) and indicates that ethanol−water mixtures have potential as mobile phases for the chromatographic fractionation of COS, differing only with respect to degree of polymerization.

The addition of small amounts of cellulose to COS containing ethanol/water solutions had a significant impact on the relative solubility of the different COS (**Figure 2**). Cellohexaose, which showed appreciable solubility in both 40 and 60% ethanol solutions in the absence of cellulose, was effectively adsorbed from these solutions upon addition of microcrystalline cellulose. The addition of microcrystalline cellulose also resulted in decreases in the cellopentaose content of the 40 and 60% ethanol solutions, although the extent of adsorption of cellopentaose was significantly less than that observed for cellohexaose. The effect of cellulose addition on cellotriose and cellotetraose solubility, in these same solutions, was negligible. The cellulose effects are consistent with the relative solubility of the different COS (*19*, *20*).

Adsorption between a solute and a adsorbent is related to the solubility of the solute. Typically, among a homologous series, adsorption increases strongly and regularly with increasing molecular weight of the solute (*21*). The cellulose component of the reaction mixtures provides a template upon which COS may adsorb (*22*). The structural similarity of the COS and cellulose surface permits strong adsorption (*23*). **Figure 2** illustrates that the adsorption of cellooligosaccharides to cellulose becomes more important with increasing chain length of the cellooligosaccharides. This is thought to be the result of an increased number of intermolecular interactions.

The chromatogram presented in **Figure 3** illustrates the potential of using water/ethanol mobile phases in conjunction with cellulose stationary phases for the fractionation of COS. The stationary phase in this case was a commercially available microcrystalline cellulose. The results demonstrate that an aqueous 20% ethanol mobile phase can be used to effectively separate the lower DP COS (DP ≤ 4) from the higher DP COS. Under these conditions, cellopentaose was obtained with baseline resolution (COS of DP ≥ 6 could not be detected, using

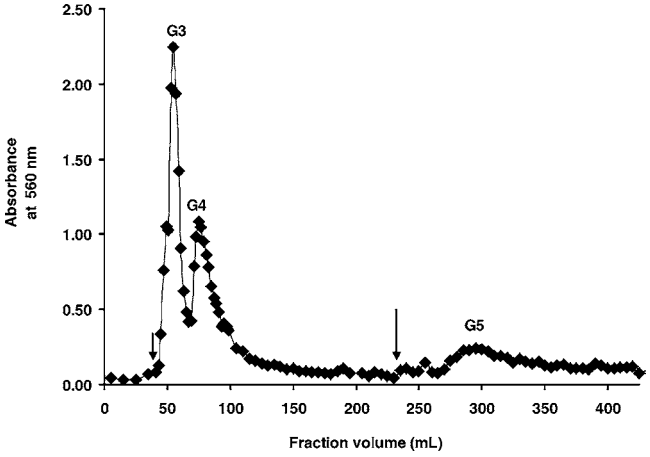

Figure 3. Elution profile of cellooligosaccharides chromatographed on microcrystalline cellulose stationary phase at room temperature. The first arrow shows the void volume in the graph. The initial mobile phase was 20% ethanol; the mobile phase was switched to water at the point corresponding to the second arrow in the figure. The predominant cellooligosaccharide in each peak is identified (G3, cellotriose; G4, cellotetraose; G5, cellopentaose).

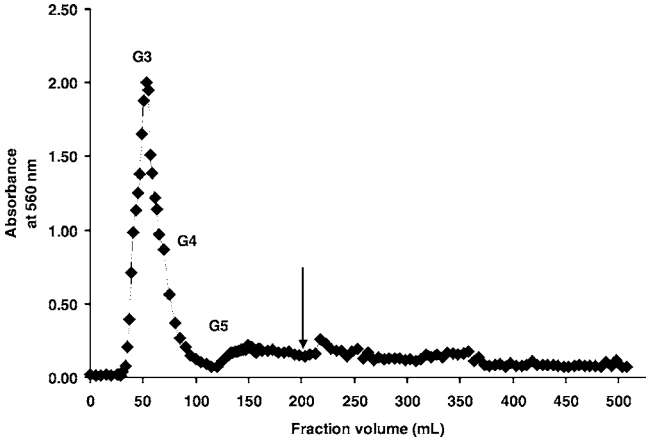

Figure 4. Elution profile of cellooligosaccharides chromatographed on fibrous cellulose stationary phase. The initial mobile phase was 20% ethanol; the mobile phase was switched to water at the point corresponding to the arrow in the figure. The predominant cellooligosaccharide in each peak is identified (G3, cellotriose; G4, cellotetraose; G5, cellopentaose).

analytical TLC and HPLC, in the G5 peak of **Figure 3**). The elution volume of the G3-labeled peak (predominantly cellotriose) was found to be greater than the void volume for the column; the implication being that COS as short as cellotriose have demonstrable affinities for cellulose stationary phases. This is in agreement with the results of Chitumbo and Brown (*23*), who observed COS-specific adsorption with cross-linked cellulose gels.

The results reported thus far were obtained using a microcrystalline cellulose as the solid phases. The question arises as to whether a less crystalline cellulose preparation would give similar results. This question was addressed by running the same experiment as depicted in **Figure 3** with the exception of using traditional fibrous cellulose as the stationary phase. The results are presented in **Figure 4**. The chromatograms of **Figures 3** and **4** are similar in that the elution orders are the same. However, retention times for the higher COS were greater with the microcrystalline cellulose. For example, cellopentaose was

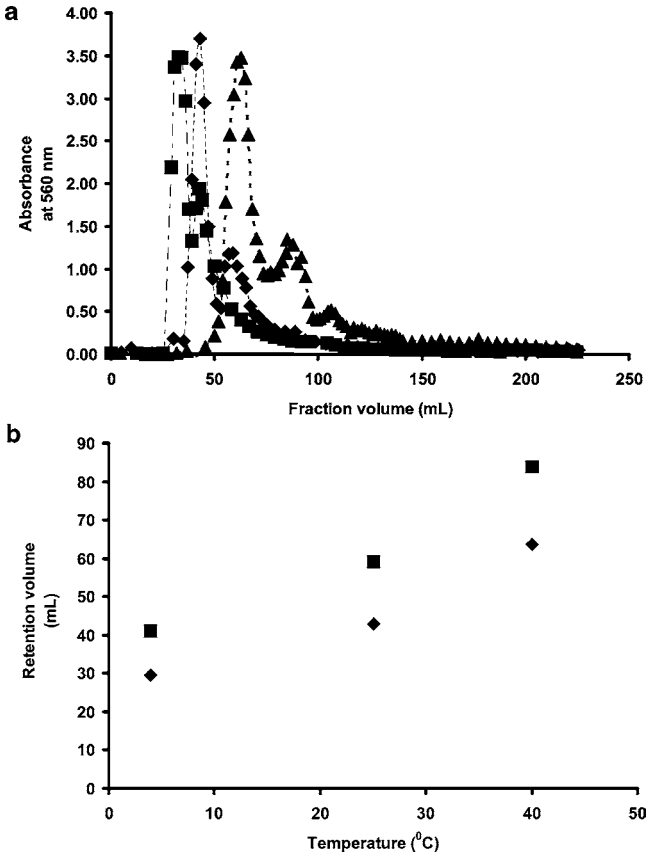

Figure 5. (**a**) Effect of temperature on elution profile of cellooligosaccharides chromatographed on microcrystalline cellulose with water as the mobile phase (4 °C, ■; 25 °C, ◆; 40 °C, ▲). (**b**) Effect of temperature on retention volume of cellotetraose (▲) and cellopentaose (■) chromatographed as in **a**.

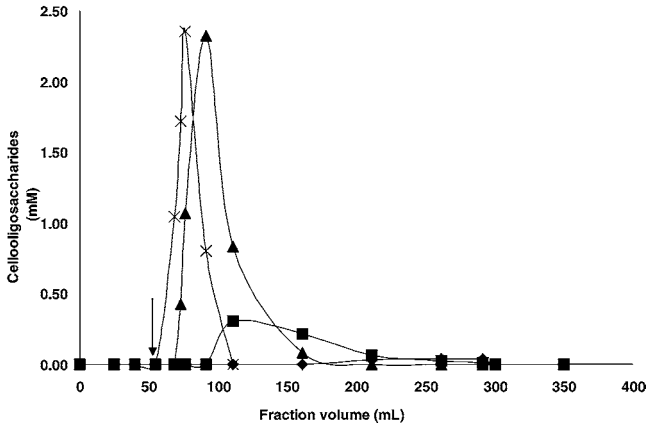

Figure 6. Elution profile of cellotriose (×), cellotetraose (▲), and cellopentaose (■) in cellooligosaccharide/cellulose precipitate obtained from 1-propanol dilution of original acid-hydrolysis solution (see Methods for details). The cellulose component of the precipitate served as the sole chromatographic medium. Cellooligosaccharides were eluted with a linear ethanol → water gradient at room temperature. The column fractions were analyzed and quantified with HPLC. The amount of each oligosaccharide found in the fractions was plotted against the elution volume.

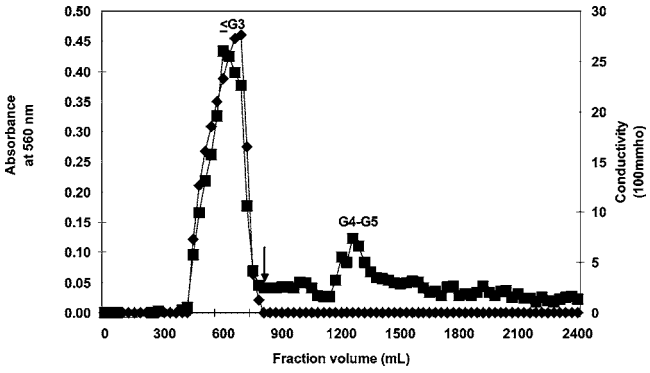

Figure 7. Elution profile of neutralized cellulose hydrolysate mixture (as reducing sugars, ■), following adjustment of soluble phase to 60% ethanol at room temperature (see Methods for details). Starting mobile phase was aqueous 60% ethanol; mobile phase was switched to water at the point corresponding to the arrow in the figure. Elution profile of the hydrolysate/neutralization salts is included (as conductivity, ◆). Predominant cellooligosaccharides are indicated on the chromatogram (G3, cellotriose; G4, cellotetraose; G5, cellopentaose).

eluted from the fibrous cellulose stationary phase, while still running the ethanol/water mobile phase; whereas it was only eluted from the microcrystalline stationary phase after switching to the water mobile phase. Thus, the more crystalline stationary phase seems to favor COS adsorption. The results indicate that the microcrystalline cellulose preparation is superior under these conditions but also that either cellulose preparation has potential for COS fractionation.

The column was tested for the potential of using water as a mobile phase in conjunction with cellulose stationary phases for the fractionation of COS (**Figure 5a**). When the column was run at 4, 25, and 40 °C with water as eluent, the first peaks contained G3 and G4, while the second peak contained G4 and G5 (predominantly G5) (**Figure 5a**). Unlike aqueous 20% ethanol mobile phase, water mobile phase at three different temperatures could not separate G3, G4, and G5 into different peaks (**Figure 3**).

The partition of solute with adsorbent is determined by the net interaction between adsorbent−solute and solute−solvent. Therefore, when the temperature is altered, it may favor or inhibit adsorption to the stationary phase (*24*). Increased temperatures corresponded with increased retention volumes for all COS. The temperature effect was slightly more pronounced for the longer COS (cellotetraose and cellopentaose), thus improving the resolving power of the column. The retention volume of the lower DP COS, which appear to have relatively low affinities for microcrystalline cellulose, also increased with temperature. It is difficult to interpret the temperature/elution volume relationship, because the porosity of the MCC stationary phase may change as a function of temperature (*25*).

Figures 6 and **7** provide data in support of how cellulose-based columns may be used for making COS preparations with differing DP profiles. In the first case, a cellulose hydrolysate, containing the complete range of COS and unhydrolyzed cellulose, was diluted with 1-2-propanol (to precipitate the COS) and the resulting liquid phase, containing the majority of the glucose and cellobiose, was subsequently removed, resulting in an alcohol-insoluble mixture containing COS plus cellulose. This mixture was then suspended in ethanol, loaded into an empty column, and washed with ethanol; the COS was eluted from the accompanying cellulose using an ethanol/water gradient. The unique aspect of this approach is that the cellulose remaining after acid-catalyzed hydrolysis is used as the stationary phase in the column. The stationary phase in this case is expected to be less crystalline than either the microcrystalline

cellulose or the fibrous cellulose, because the dissolution process preceding cellulose hydrolysis is similar to that used for the preparation of amorphous cellulose (*26*). The chromatogram of **Figure 6** illustrates that two crude COS preparations, one dominated by cellotriose (50−75 mL eluate) and the other dominated by higher DP COS (>75 mL eluate), could be obtained. Interestingly, the higher DP COS were eluted from this column at higher ethanol concentrations than with the corresponding columns discussed above. This is consistent with the higher DP COS having a greater affinity for crystalline cellulose.

In the second case, the liquid phase resulting from the cellulose hydrolysis process (see "Methods") is neutralized, made 60% in ethanol, and then directly applied to a cellulose column. Hence, the solution to be chromatographed contained substantial amounts of glucose and cellobiose, these being unavoidable side-products generated during the making of COS via acid-catalyzed hydrolysis. These sugars are not present to any significant extent in the previously discussed COS preparations because they were removed, prior to chromatography, by repeated alcohol washes (*18*). In the present case, the complete mixture resulting from the hydrolysis of cellulose was chromatographed. It can be seen that the glucose and cellobiose, along with the neutralizing salts, were eluted from the column in the initial carbohydrate-containing fractions; the higher molecular weight COS coming in later fractions (**Figure 7**). Hence, the data illustrates the potential of using cellulose columns, as a possible alternative to alcohol washes (*18*), for the separation of lower molecular weight sugars from COS. This type of separation is expected to be a prerequisite step for many applications of COS. The implementation of this type of chromatography in industrial processes would necessarily involve appropriate scale-up considerations.

The combined experiments presented herein demonstrate the potential of using cellulose stationary phases for the separation of water-soluble COS. In such systems, the retention times/volumes for COS of DP three through six are shown to correlate with their relative solubility in aqueous ethanol solvents.

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