



SCIENTIFIC AND TECHNICAL JOURNAL  
Namangan Institute of Engineering and Technology

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POLYSACCHARIDE DERIVATIVES FROM NATURAL SOURCES»

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<https://doi.org/10.5281/zenodo.7951017>



ISSN 2181-8622

**Manufacturing technology problems**



**Scientific and Technical Journal  
Namangan Institute of  
Engineering and Technology**

**Volume 8  
Issue 1  
2023**



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## SIZE-EXCLUSION CHROMATOGRAPHY OF SOME POLYSACCHARIDE DERIVATIVES FROM NATURAL SOURCES

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### Abstract:

**Objective.** In the article molar mass and structural properties some of natural polysaccharides and their derivatives were studied by Size-exclusion chromatography.

**Methods.** For investigation of electrostatic and polyelectrolyte properties of natural polysaccharides Exclusion liquid chromatography method was used.

**Results.** Polyelectrolyte and electrostatic effects of polysaccharide derivatives in Size-exclusion chromatography were suppressed by using of aqueous eluent containing salt solution.

**Conclusion.** As a result of the research, it was shown that many of polysaccharides are polyelectrolytes and determination of their molar mass parameters is complicated by electrostatic effects in Size-exclusion chromatography.

**Key words:** polysaccharides, carboxymethyl chitosan, galactomannan, exclusion chromatography, heparin, electrostatic effects, polyelectrolytes.

**Introduction.** Water-soluble derivatives of polysaccharides are widely used due to a wide range of their useful and unique properties in biomedicine, pharmaceuticals, cosmetology, agriculture, and other fields. Biologically active polysaccharides from natural raw materials include chitosan, carrageenan, arabinogalactan (AG), heparin, agar, fucoidan, etc. Agar and carrageenan are obtained by extraction from red and fucoidan from brown seaweeds, and the chains of green algae polysaccharide molecules have a heterogeneous structure with sugar residues, such as glucuronoxylogamannans, glucuronoxylo-

rhamnogalactans, and xyloarabinogalactans [1, 2]. They are considered potential biologically active substances and have immunomodulatory, antitumor, antiviral, and antibacterial properties [3]. The most widely used anticoagulant drug in modern medical practice is the natural glycosaminoglycan heparin, however, the use of heparin causes some side effects, such as bleeding, and heparin-induced thrombocytopenia. The anticoagulant activity of sulfated polysaccharides

depends on the method of sulfation, which affects the degree of sulfation, the nature, and location of sulfate groups, molecular weight, etc. Many sulfated polysaccharides have a variety of biological activities, including anticoagulant, antithrombotic, antiviral, and antibacterial. [4-8]. Plant polysaccharides such as pullulan, galactan, galactomannan, and fucoidan sulfates have anticoagulant activity [9–13]. This article discusses the physicochemical and molecular weight characteristics of polysaccharides and their derivatives determined by the method of Size Exclusion Chromatography (SEC).

It is known from a few literature sources [10, 13] that sulfated derivatives of AG have hypolipidemic and anticoagulant activity. In the laboratory of natural synthons and ligands of the Irkutsk Institute of Chemistry, named after A.E. Favorsky Siberian Branch of the Russian Academy of Sciences, sulfated AG was obtained in the form of a potassium salt by sulfating AG with the SO<sub>3</sub>-dimethylformamide complex in dimethyl sulfoxide [11, 12]. In preliminary preclinical studies, the drug proved to be a promising lipid-lowering agent with a pronounced anticoagulant effect [14–17]. A relatively small number of works [18, 22] have been devoted to the study and determination of the molar mass of AG. Thus, in [18], the molecular weight distribution (MWD) of larch AG samples was determined by the SEC method on an Agilent 1200 chromatograph with a 1260 Infinity refractometric detector (30°C), PL Aquagel-OH 40 300 × 7.5 mm, 0.1 M LiNO<sub>3</sub>, and 1 mL/min). The column was calibrated using standard samples of dextran (Sigma-Aldrich) with molecular masses of 10600, 20000, 41272, and 70000 Da. As the feedstock, we used AG obtained by the original method [18] from Siberian larch wood (*Larix sibirica* Ledeb.). The infrared spectra and MWD of arabinogalactan sulfates in the form of sodium and ammonium salts, obtained using various sulfating reagents, were compared. According to the data obtained,

the investigated sulfated derivatives and the initial samples of AG differ noticeably in terms of hydrogen bonds and molar mass distribution. In [23], the synthesis of guar gum sulfates by a complex of sulfur trioxide with 1,4-dioxane was studied. It is shown that the following optimal conditions for sulfation of guar gum with sulfur trioxide-1,4-dioxane complex were established: temperature 60 °C, duration 2.9 h, and volume of chlorosulfonic acid 3.1 ml. The presence of sulfate groups in the structure of guar gum was confirmed by elemental analysis and Fourier transform infrared spectroscopy (FT-IR). Sulfated guar gum has also been characterized by X-ray diffraction analysis, scanning electron microscopy, and gel chromatography. From gel chromatography data, it was shown that during the sulfation of guar gum with a sulfur trioxide complex with 1,4-dioxane, the molecular weight decreases from 600 to 176 kDa.

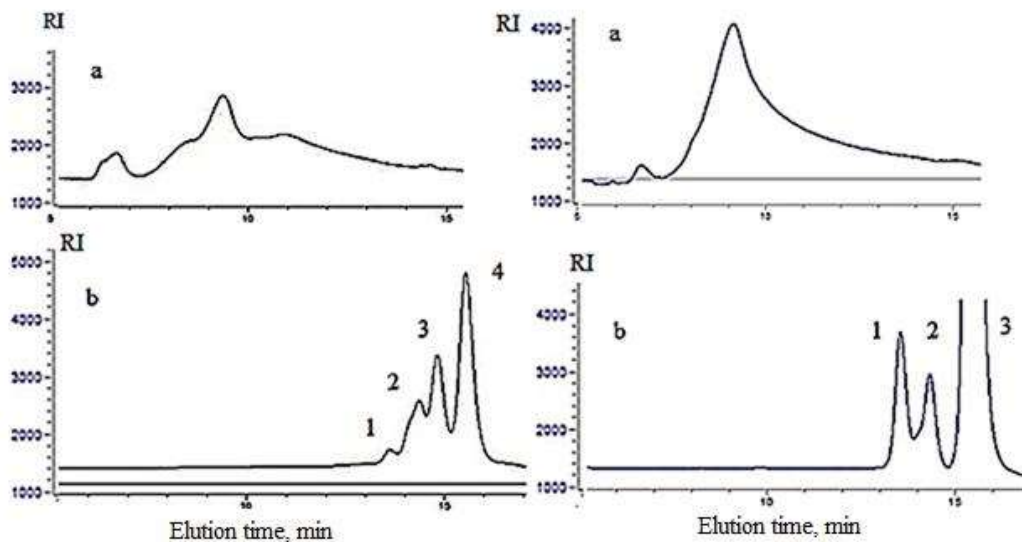
Sulfated derivatives of the galactomannan family have a variety of biological activities. In [24], sulfated galactomannan was obtained from fenugreek gum in a chlorosulfonic acid/pyridine medium. To obtain derivatives with the highest degree of substitution (DS), the optimal conditions for sulfation were determined in the experiment according to the Box-Behnken scheme. Analysis of the quadratic regression model confirmed that reaction time was the most significant DS exposure parameter. Under the chosen conditions, the maximum value of the degree of sulfation was obtained at 0.490. The results of FT-IR and X-ray photoelectron spectroscopy (XPS) showed the presence of the SO<sub>3</sub>- group. In <sup>13</sup>C NMR spectroscopy, the original C-6 peaks did not completely disappear, and new peaks appeared at  $\delta$  63.2 and 64.0, illustrating incomplete substitution, predominantly in the C-6 position. After sulfation, Size exclusion chromatography combined with polygonal laser light scattering (SEC-MALLS) found that the average molecular

weight ( $M_w$ ) of the sulfated derivatives rapidly decreased. The introduction of negatively charged  $\text{SO}_3^-$  groups into the electrostatic interaction and the decrease in MM could have a significant effect on its biological activity [24].

The extraction of carboxymethyl chitosan (CMCHT) from chitosan (Sigma-Aldrich, USA) was carried out as follows. 1.0 g of chitosan was mixed in 25 ml of isopropyl alcohol for 10 minutes at room temperature, and 8 ml of 40% NaOH was added to the suspension. As a result, the suspension was brought to a standstill. After that, another 35 ml of isopropyl alcohol was added to the solution and stirred for 30 minutes at room temperature. After mixing the suspension well, adding 5 g of monochloroacetic acid, the temperature of the solution was raised to

450 °C and stirred for 3 hours. After that, the solution was cooled to room temperature and filtered. After filtration, it was washed with 200 ml of methanol. Then, the sediment was removed from the filter paper and put into a 200 ml beaker, 100 ml of methanol was poured over it, and 10 drops of acetic acid were added. It was covered with foil and stirred at room temperature for 14 hours. Mixing was stopped, and the solution was cooled for 10 minutes. Then the solution was filtered, and the filter was washed with ethyl alcohol 3–4 times. A small sample was taken and dissolved in water to check solubility. The pH value of the dissolved solution was determined to be neutral. The resulting wet precipitate was dried at 500 °C for 12–14 hours and weighed 2.1 grams.

SEC analysis of synthesized CMCHT was carried out in water (Fig. 1a and 2a)



**Fig.1**

**Fig.2**

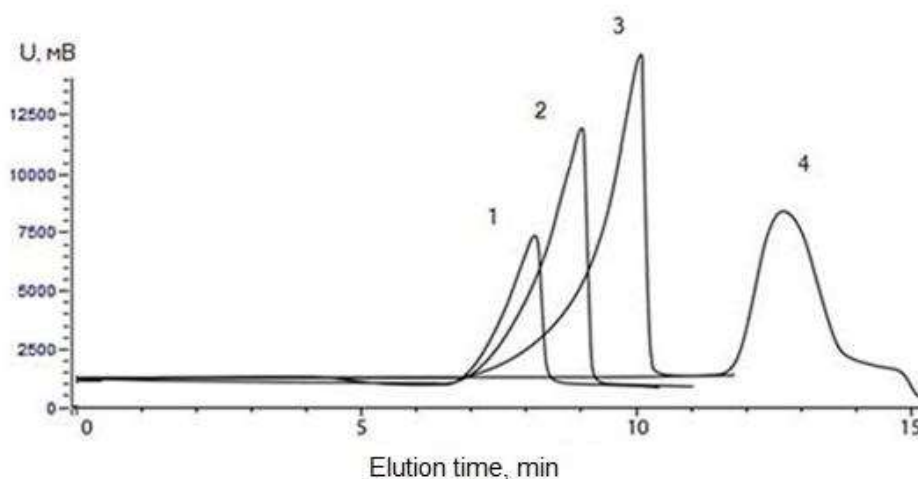
Elution chromatograms of carboxymethyl chitosan from Shandong Yinuokang

Pharmaceutical Co., Ltd (China) (Fig.1) and from Black Sea crab (Turkey) (Fig.2). and salt solutions as eluent (Fig.1 b and 2b). Figures 1 and 2, respectively, show the chromatograms of CMCHT obtained from chitosan synthesized by the Chinese Shandong Yinuokang Pharmaceutical Co., Ltd. and CMCHT

synthesized by modifying chitin, extracted from the shell of the Black Sea crab (Turkey). The chromatograms presented in Figures 1a and 2a were recorded when water was used as an eluent in SEC, and we can see that the properties of CMCHT molecules describe the polyelectrolyte expansion effect, i.e., the reduction of the

elution time and the multimodal appearance of the elution curves. The reason for this is that, due to the ionization of the carboxyl groups in the CMCHT chain in water, anions move away from each other under the influence of Coulomb forces, that is, macromolecules grow in geometric size. This phenomenon is called the polyelectrolyte expansion effect. Chromatographic peaks with small values occur due to the increase in the size of macromolecules and their early exit from the column before being able to enter the pores on the surface of the sorbent. This anomalous phenomenon was eliminated when a solution of  $\text{NaNO}_3$  in water with a concentration of 0.1 mol/l was used as an eluent. As a result of the screening (blocking) of anionic groups by  $\text{Na}^+$  ions in water, the forces of electrostatic interaction decrease, and the polymer chain becomes neutral. In this case, the elution time corresponding to the peaks of the chromatograms increases, and the symmetry of the chromatograms becomes visible. This situation indicates that the molecular sieve separation mechanism of

SEC is activated (Figures 1b and 2b). As can be seen from Figures 1b and 2b, the samples consist of 4 and 3 fractions, respectively. Fractions 1, 2, 3 in Figure 1 b belong to the CMCHT sample, and peak 4 belongs to the solvent. In Fig. 2b, it was found that fractions 1, 2 belong to CMCHT, and peak 3 belongs to the solvent. SEC was used to study the polyelectrolytic nature and determine the average molecular weights of commercial unfractionated heparin "Heparin-Indar" (Ukraine) and low molecular weight heparin Clexane (Sanofi, France). SEC was performed on an Agilent 1260 Infinity high speed liquid chromatograph (USA) with a refractive index detector. The eluent flow rate was 0.8 ml/min. The volume of the injected sample was 25  $\mu\text{l}$ . Figure 3 shows combined gel chromatograms of low molecular weight heparin brand "Clexane"(SANOFI-AVENTIS FRANCE), obtained at different concentrations of the injected sample in water (curves 1,2, 3) and in an aqueous solution of 0.1 M  $\text{NaNO}_3$  (curve 4).

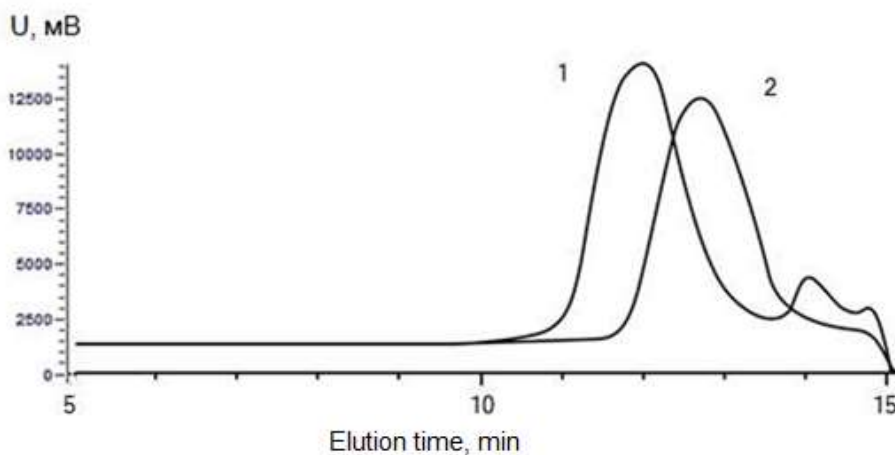


**Fig.3. Overlapped chromatograms of heparin "Clexane" in water (1,2,3) and salt solution (0.1M  $\text{NaNO}_3$ ) (4). Concentrations at peaks, g/dl: 1 – 0.05; 2 -0,1; 3-0.2**

It can be seen from the figure that, without the addition of neutral salt or water, the chromatograms have asymmetric shapes, and the retained volumes (or elution time) decrease with decreasing

polymer concentration in the solution, which characterizes the presence of the polyelectrolyte swelling effect in the chromatographic system. With the use of an aqueous solution having the neutral salt

( $\text{NaNO}_3$ ) with a concentration of 0.1 mol/l as an eluent, electrostatic effects were eliminated. It can be seen from Fig.4, where separation of two commercial heparins: Heparin-Indar (Ukraine) and low molar mass heparin "Clexane".



**Fig.4. Overlapped chromatograms of Heparin-Indar (M=15 kDa) (1) and low molar mass heparin "Clexane" (M=4.5 kDa) (2)**

Note that the suppression of the electrostatic effects of the samples during the SEC process occurs due to the screening of the Coulomb repulsive forces of sulfate groups in the chains of heparin molecules due to the presence of  $\text{NaNO}_3$  salt in water.

**Conclusion.** In the SEC of anionic polysaccharides, such as carboxymethyl chitosan and heparin, the separation

mechanism in pure water as eluent is distorted by the polyelectrolyte expansion effect. In these cases, elution profiles of chromatograms will have an asymmetric form, and retention times (volumes) will decrease at small concentrations of injected solutes. In eluent containing 0.1 moles/l of  $\text{NaNO}_3$ , electrostatic effects were suppressed.

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