Universal calibration for natural polysaccharides in aqueous Size-exclusion chromatography

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Abstract. This article shows the validity of the Universal calibration concept in aqueous Size-exclusion chromatography for arabinogalactan and carboxymethylchitosans is presented. The electrostatic effects of these polysaccharides during separation process were eliminated by utilizing of a water-salt eluent as a mobile phase, and the size-exclusion separation mechanism of this method was realized.

1 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, heparin, agar, fucoidan, etc. Chitin is a linear polysaccharide consisting of elementary units of 2-acetamido-2-deoxy-D-glucose connected by 1,4-β-glycosidic bonds. The chitin derivative, chitosan, can be referred to as a biologically active polysaccharide [1]. Chitosan is one of the most promising polymers for biomedicine and pharmaceuticals due to its unique properties. It has great potential due to its high adsorption capacity, biodegradation, biocompatibility, and low toxicity. The biological activity of carboxymethylated derivatives of chitin and chitosan depends on the method of carboxymethylation of hydroxyl and amino groups, which allows modification of the chitosan molecule by introducing various functional substituents, obtaining derivatives with different properties, which significantly expands the scope of this biopolymer, due to the possibility of affecting the degree of substitution, nature, and location of carboxymethyl groups, as well as their molar mass (MM). Special attention is devoted to carboxymethyl chitosan (CMCh), obtained by the carboxymethylation of chitosan. One of the important properties, of MM is that it significantly affects the biological activity of CMCh. The presence of hydroxyl and amino groups makes it possible to modify the chitosan molecule by introducing various functional substituents, and obtaining derivatives with different properties, which significantly expands the scope of this biopolymer, due to the possibility of directed changes in its properties.

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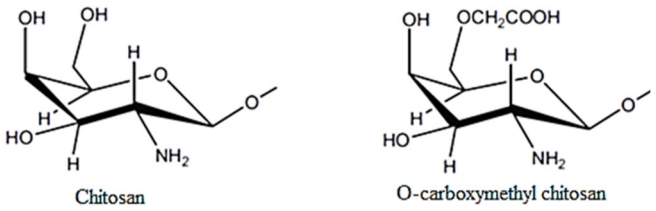

Fig. 1. Schematic presentation of chitosan and carboxymethyl chitosan.

Arabinogalactan (AG) is a natural polysaccharide of plant origin. In significant quantities (10 ... 15%) it is contained in larch wood, which can serve as a source of its production. AG has very good prospects for use, for example, as a thickener, stabilizer, filler, coating agent [2]. A significant influence on the quality of this polymer as a commodity product (from adhesive to medical preparations) is exerted by its molecular weight. Chemically, AG from larchwood have a general structure given by a backbone of (1 → 3)-linked β-d-galactopyranosyl units that account for about one-third of the molecule, each of which contain a side chain at position C6. Most of these side chains are galactobiosyl units containing a (1 → 6)-β-d-linkage. Another side chain type that occurs is a single l-Ara unit or 3-O-(β-l-arabinopyranosyl)-α-l-arabinofuranosyl units. The arabinogalactan macromolecule from larch wood has a highly branched structure. Its main chain consists of galactose units connected by glycosidic bonds, and the side chains of galactose and arabinose units, of single units of arabinose, as well as uronic acids, mainly glucuronic. There is evidence that arabinose units are also present in the main chain of the macromolecule. The ratio of galactose and arabinose units is approximately 6: 1, while 1/3 of the arabinose units is in the pyranose form, and 2/3 is in the furanose form. These ratios, as well as the molecular weight of AG, can vary depending on the species of larch, as well as within the same species. The composition of AG macromolecules also varies depending on the conditions for its isolation from wood and molecular weight. In [3] stated that in water, AG is capable of intermolecular interaction and possibly exhibits polyelectrolyte properties, due to which the molecular weights determined by gel chromatography are overestimated. Determination of the molecular weight of AG must be carried out under conditions of destruction of associates and suppression of polyelectrolyte effects using complex systems of eluents, consisting, in particular, of phosphoric acid and lithium bromide, for which optimal concentrations are selected.

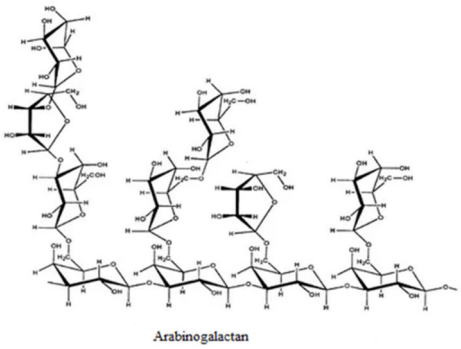

Fig. 2. Chemical structure of arabinogalactan.

Size-exclusion chromatography (SEC) or Gel permeation chromatography (GPC) is one of the powerful and fast methods for the determination and characterization of the average molar mass of polymers [4]. The chromatographic behavior of macromolecules separated by

SEC can be described by the general chromatographic equation: $Kd = (V_R - V_0) / (V_t - V_0)$, where V_R is the measured peak elution volume, Vt the total column volume, and V_0 the exclusion (or void) volume. The concept of UC in GPC was introduced by Benoit in 1967 [5]. Instead of plotting the log molecular weight of a series of narrow polymer standards vs. retention volume, the logarithm of the product of the intrinsic viscosity [η] and molecular weight M is plotted vs. retention volume. The product (M[η]) has dimension unit dl/mole and can characterize the hydrodynamic volume (Vh) of macromolecules. The GPC plot between log (M[η]) and V_R, represents a universal calibration of Benoit and retention volume V_R can be determined from the following equation: $V_R = A - B \lg (M[\eta])$, where A and B - constants. Using UC constructed for polymers having different conformation in solution (random coil, rodlike, branched, star, copolymers, etc) and their average molar masses can be determined. This study aims to analyze the applicability of the UC to pullulans, arabinogalactans (AG) and CMCh in aqueous solutions.

2 Experimental research

GPC measurements were performed on the liquid chromatograph Agilent 1260 Infinity, consisting of a syringe pump, differential refractive index (RI) detector, and degasser of the eluent. For aqueous GPC the chromatographic column PL Aquagel-OH Mixed (300x8 mm, Polymer laboratories, UK) thermostated at 25^0C was used. GPC analysis was performed using $NaNO_3$ in the water with a concentration of 0.1 mol/l as eluent. Pullulan standards were purchased from Showa Denko, Japan. Larch AG was provided by Megazyme Int, Ireland. The injection volume of the sample and the flow rate of eluent was equal to 20 microliters and 0.8 ml/min. correspondingly. The injected sample concentration of polymers was always equal to 3 g/l. Elution volumes were determined at the peak maximum of GPC curves, since all chromatograms obtained in aqueous salt solutions gave quite sharp, symmetric curves of Gaussian type. The extraction of CMCh from chitosan (Sigma-Aldrich) 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. The suspension was mixed by adding 5 g of monochloroacetic acid, the temperature of the solution was raised to 45^0C and stirred for 3 hours. After that, the solution was cooled at room temperature and filtered and then, the suspension was washed with 200 ml of methanol. Then, the sediment was removed from the filter paper and put into a 200 ml beaker, and 100 ml of methanol was poured over it, and 10 drops of acetic acid were added to the filtrate, covered with foil and stirred at room temperature for 14 hours. Mixing was stopped and the solution was cooled for 10 minutes, was filtered and the filtrate was washed with ethyl alcohol 3-4 times. A small amount of sample was taken and dissolved in water to check solubility. The pH value of the dissolved solution was neutral.

3 Results

Electrostatic interactions in the polymer chain in GPC are manifested in a change in the retention volume (or elution time) with changing in the concentration of polymer in the sample. Such anomalous behavior of polyelectrolytes is explained by the fact that the dilution of their solutions leads to a decrease in the screenings of electrical charges in the circuit. If the Coulombic electrostatic repulsion forces inside the macromolecule will increase, then the polymer chain swells, and the size of the macromolecules increases. The polyelectrolyte expansion effect in GPC is manifested in a decrease in the retained volumes with a decrease

in the concentration of polymer in the sample. Such anomalous behavior of polyelectrolytes is explained by the fact that the dilution of their solutions leads to reducing the screenings of electric charges in the chain. AG is a water-soluble natural polysaccharide having a small amount of glucuronic acids and in pure water, its chain will cause a polyelectrolyte expansion effect [7]. The process of polyelectrolyte expansion can be seen from combined chromatograms of larch AG in pure water at different injected concentrations (lines a, b, c) and water-salt eluent (line d) that is presented in Figure 3.

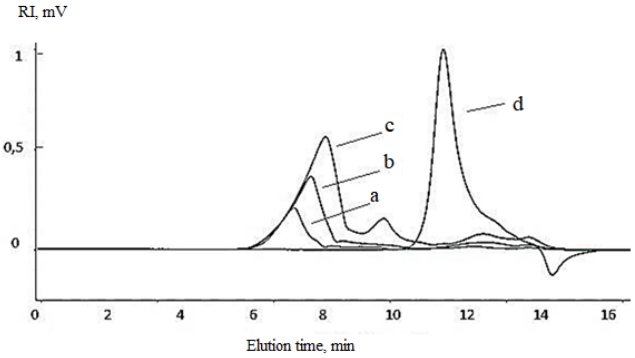

Fig. 3. Refractive Index (RI) detector response from elution time dependence in different injected concentrations of AG in water (mg/ml): a) 1; b) 2; c) 4 and water + 0,1 M $NaNO_3$ as mobile phase d).

With decreasing injected polymer concentrations, asymmetric elution profiles and reduced retention volumes confirm the existence of electrostatic effects in this system (lines a.b,c). When 0.1 M NaNO3 in H_2O is used as the mobile phase, polyelectrolyte effects in the GPC of AG are eliminated (line d). Using a water-salt eluent, we evaluated the molar mass distribution of AG composites with metals in a previous GPC analysis [7]. The goal of such tests was to verify the GPC separation mechanism and determine AG's molar mass characteristics, not to test the validity of the UC. In Figure 4 UC is shown for AG and pullulans in the molecular weight range of (1 – 1 10^3 kDa) have been obtained in 0,1 M $NaNO_3$. The Mark-Kuhn-Houwink-Sakurada constants data for the above-mentioned polymers were used for the construction of UC of Benoit.

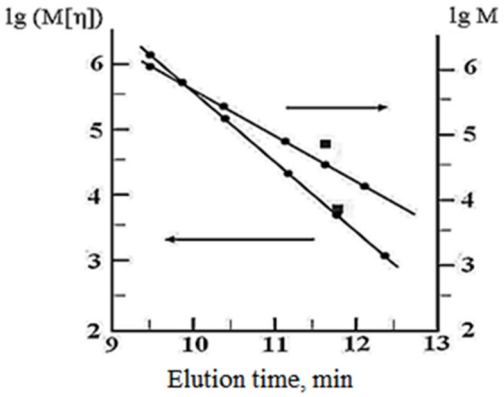

Fig. 4. Combined dependences lg (M[η]) and lg M from elution time (universal and primary calibration correspondingly) for Pullulan standards (●) and larch arabinogalactan (Megazyme, Ireland) (■). Column: PL Aquagel OH Mixed. Mobile phase: water + 0.1 M $NaNO_3$.

Molar mass and polydispersity were determined using UC, and chromatograms were evaluated according to the size-exclusion separation mechanism of GPC. According to UC

two polymers with the same hydrodynamic volume (Vh ∞ [η]M) will be separated with the same elution time Vt. The use of (M[η]) as a universal parameter representing the hydrodynamic volume of polymer chains in good solvents was proposed by various authors [8, 9]. Using $Vh_1=Vh_2$, $Vh_1= [\eta]_1M_1$ (for pullulans), $Vh_2= [\eta]_2M_2$ (for AG), and Mark-Kuhn-Houwink-Sakurada equation ($[\eta]=KM^a$) for both polymers we can determine molar mass for AG at fixed elution time that point lies on UC curve (Figure 4). From equality of hydrodynamic volumes for both polymers $K_1M_1(1+a1) = K_2M_2(1+a2)$ molar mass of AG can be determined: $\lg M_2 = \lg (K_1/K_2) / (1+a_2) + \lg M_1 (1+a_1) / (1+a_2)$. Aqueous GPC measurements indicated that pullulans (standards) and larch AG in water-salt solution (0.1M $NaNO_3$) as eluent demonstrated the validity of UC for both polymers. It should be noted that AG has a small number of galacturonic acids in its chain dissociating to anionic groups in pure water. Before construction UC for AG and pullulans polyelectrolyte expansion effect of AG was suppressed by using the aqueous salt solution as eluent. Figure 4 represents overlapped universal and primary calibrations for pullulan standards and larch AG after eliminating electrostatic effects. As shown in Figure 4 point for AG lies on the curve lg (M[η]) from retention time (left curve). For the construction of UC, we used the following constants of Mark-Kuhn-Houwink's equation for pullulans: $K_1= 1.91\ 10^{-4}$ dl/g and $a_1= 0.67$; for AG: $M_2= 42$ kDa [3] and $[\eta]_2 = 0.1$ dl/g (data from Megazyme, Ireland). For the determination of the molar mass of CMCh, the following constants of the Mark-Kuhn-Houwink equation were used: $K= 7.92\ 10^{-5}$ dl/g and a= 1[10]. Figure 5 chromatograms of CMCh received in pure water (a) and salt solution (b) as eluents are presented. As CMCh in pure water shows polyelectrolyte properties, asymmetric and multimodal profiles of chromatograms were recorded by the Refractive index detector.

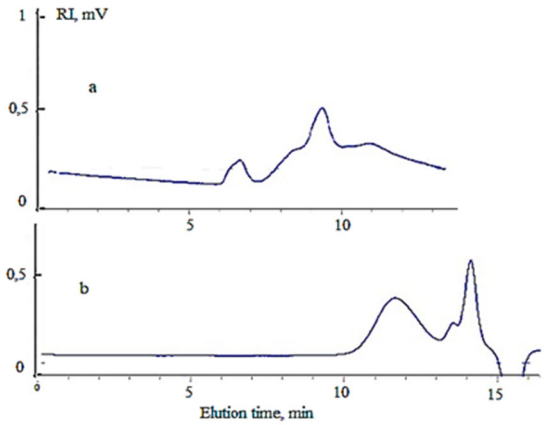

Fig. 5. Size-exclusion chromatograms of carboxymethyl chitosan. Eluent: pure water (a) and 0,1 M $NaNO_3$ in water (b). Column: PL Aquagel OH Mixed.

When we used aqueous 0,1 M NaNO3 in water the chromatograms became symmetrical profile that indicated suppressing of electrostatic effects. Multimodal asymmetric chromatograms of CMCh in pure water (Figure 5 a) can be explained by the following. The concentration of polymer molecules corresponding to the leading and trailing edge of the gel-chromatogram is always smaller than the concentration of molecules at the peak maximum. When CMCh molecules are moving through the chromatographic column, the hydrodynamic volume (or size) of molecules at the front and trailing edge of the chromatogram will be increased due to intramolecular electrostatic interactions (polyelectrolyte expansion). In this case, molecules will move fastly and elution time will be decreased. As result, asymmetrical profiles of peaks will appear and the front edge of the chromatogram becomes strongly elongated. The back of the chromatogram acquires a sharp decline. The molecules that meet

the rear of the chromatogram are also moved faster than molecules corresponding to a maximum the peak.

4 Conclusions

We confirmed the validity of the UC principle for pullulans, AG, and CMCh in aqueous salt solutions on the PL Aquagel OH Mixed column in GPC. CMCh has carboxylic acids and AG a small number of galacturonic acids in the polymer chain that dissociates into anions in water. Due to of Coulombic intramolecular electrostatic repulsion forces of anionic groups the molecular sizes of polymer chains will be increased. The use of aqueous 0,1 mol/l sodium nitrate allows the elimination of these electrostatic effects like polyelectrolyte expansion, and any enthalpic polymer-substrate interactions. The validity of UC for pullulans, AG, and CMCh is realized in the aqueous salt eluent.

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