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QUANTIFICATION OF THE COMPOSITION OF GUM ARABIC-CHITOSAN COACERVATES BY HPLC

CUANTIFICACIÓN DE LA COMPOSICIÓN DE COACERVADOS DE GOMA ARÁBIGA-QUITOSANO POR HPLC

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Abstract

Formation of a complex coacervate between gum Arabic and chitosan produces a spontaneous separation into a coacervate (precipitated) phase coexisting with an equilibrium (soluble) phase. The relative concentrations between the polysaccharides in these two phases, is difficult to quantify. In this work, the equilibrium phase was subjected to acid hydrolysis, and evaluation of the produced monosaccharides was done by HPLC. Composition of the coacervate phase was computed by mass balance. These results were compared with those obtained previously for the same system, where the coacervate phase was analyzed by elementary analysis. Results obtained using both procedures were non-significantly different.

Keywords: gum Arabic, chitosan, complex coacervation, acid hydrolysis, HPLC quantification, equilibrium phase.

Resumen

La formación de un coacervado complejo entre la goma arábiga y el quitosano produce una separación espontánea en una fase coacervada (precipitada) que coexiste con una fase en el equilibrio (soluble). No es una tarea sencilla la determinación de las concentraciones relativas entre los polisacáridos en ambas fases. En este trabajo se le efectuó una hidrólisis ácida a la fase en el equilibrio, y se cuantificaron los monosacáridos resultantes por HPLC. La composición de la fase coacervada se determinó por balance de masa. Los resultados obtenidos se compararon con aquellos obtenidos para el mismo sistema utilizando análisis elemental, sin que se hayan encontrado diferencias significativas por ambos métodos.

Palabras clave: goma Arábiga, quitosano, coacervación compleja, hidrólisis ácida, cuantificación por HPLC, fase en el equilibrio.

1. Introduction

Polyelectrolytes are polymers that develop substantial charge when dissolved or swollen in a highly polar solvent such as water (Walstra, 2003). Polysaccharides are known as biomacromolecules polyelectrolytes or polyions because their charge arises from many ionized functional groups positioned along the backbone, while proteins are known as polyampholytes, because they contain simultaneously positive and negative charge units in their backbone. Most of the polysaccharides are anionic polyelectrolytes as Arabic, mesquite, carrageenan, xanthan, guar, and gellan gums, etc. Chitosan is the only cationic pseudonatural polysaccharide (Rinaudo *et al.*, 2005) and for this reason chitosan complexation with anionic polysaccharides (Sankalia *et al.,* 2007; Espinosa-Andrews *et al.,* 2007; Fredheim and Christensen, 2003) or proteins (Montilla *et al.,*2007; Guzey and McClements, 2006) has been an ongoing research topic. The interaction between oppositely charged polyelectrolytes leads to the formation of soluble or insoluble complexes. Complex coacervation involves spontaneous separation into coexisting solvent-rich

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and solvent-depleted phase, the latter consisting of a coprecipitate of both biopolymers (Dickinson, 1995).

However, the quantification of the concentration of the biopolymers in equilibrium in these complexes is a difficult task. When complex coacervation occurs between a polysaccharide and a protein, the quantification usually relies on first determining the protein concentration by a suitable method and afterwards that of the polysaccharide. High performance liquid chromatography (HPLC) with a selective proteins column has been used to quantify protein concentration in protein-polysaccharide complexes, achieving an efficient separation and quantification of the proteins from the complex (Weinbreck *et al.,* 2003; Bohidar *et al.,* 2005; Schmitt *et al.,* 2005). The interaction between *β*-lactoglobulin and chitosan has been studied by several authors. In one work *β*-lactoglobulin concentration was determined using a modified Lowry method, whereas chitosan concentration was determined by quantifying the hexosamines (Guzey and McClements, 2006). In another work *β*-lactoglobulin concentration was estimated by reversed phase-high performance liquid chromatography and chitosan by glucosamine quantification by gas chromatography after acid hydrolysis (Montilla *et al.,* 2007).

When complex coacervation occurs between polysaccharides, quantification of the relative concentrations between the polysaccharides must be determined. The composition of the coacervate phase between chitosan and lignosulfonate was determined by quantifying the nitrogen/sulphur ratio using 2,5-bis(5-*tert*-butyl-benzoxazol-2-yl)thiophen as standard for both sulphur and nitrogen by elemental analysis (Fredheim and Christensen, 2003). The relative concentrations of gum Arabic and chitosan in coacervate phases obtained by mixing different gum Arabic-chitosan ratios by elementary analysis was studied (Espinosa-Andrews *et al.,* 2007). Total C, H, and N were determined in the coacervate phases, and for each of the pure biopolymers. N was selected for determining the mixture composition because it provided a reasonable element differential between gum Arabic and chitosan. Equilibrium phase composition was determined by mass balance. Nevertheless, due to the high operational cost and the relatively low availability of elemental analysis equipment in many laboratories, it is convenient to explore the use cheaper and more widespread available alternative methods for determining the relative polysaccharide concentrations in binary complex coacervation blends.

The aim of this work was to establish a reliable HPLC method for determining the relative equilibrium concentrations of gum Arabic and chitosan in complexes obtained under different mixing ratios.

2. Materials and methods

2.1 Chemicals and reagents

Chitosan (medium molecular weight, degree of deacetylation: 79%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chitosan is the second polysaccharide most abundant in the world and is obtained by alkaline *N*-deacetylation of chitin. Chitosan is a heterogeneous binary polysaccharide that consists primarily of 2-acetamido-2-deoxy-*β*-D-glucopyranose and 2-amino-2-deoxy-*β*-D-glucopyranose residues, the latter residue being responsible for its cationic charge at acidic pH values (Claesson and Ninhami, 1992). Gum Arabic (*Acacia senegal*) tear drops were purchased from Industrias Ragar, S.A. de C.V. (Mexico City, Mexico) and was purified as indicated by Loeza-Corte *et al.* (2007). Gum Arabic is a branched, neutral or slightly acidic, complex polysaccharide obtained as mixed calcium, magnesium, and potassium salt (Renard *et al.,* 2006). Studies on the structure of gum Arabic indicate that the molecules consist of a β 1→3 linked galactopyranose backbone chain with numerous branches linked through β 1→6 galactopyranose residues and containing arabinofuranose, arabinopyranose, rhamnopyranose, glucuronic acid and 4-*O*-methyl-D-glucuronic acid, with small amount of proteinaceous material as an integral part of the structure (Williams *et al.,* 1990; Renard *et al.,* 2006; Vázquez-Ortiz *et al.,* 2006; Osman *et al.,* 1993). All reagents used were of analytical grade purity. D-glucuronic acid, L-arabinose, D-galactose, and L-rhamnose standards were purchased from Sigma (St. Louis, MO, USA). Sulphuric acid and barium bicarbonate were purchased from J.T. Baker (Xalostoc, State of Mexico, Mexico).

2.2 Sample preparation

The samples were prepared as reported by Espinosa-Andrews *et al.* (2007). A chitosan stock solution (2.0% wt in 1.0% wt acetic acid) and a gum Arabic stock solution (20% wt in water) were made. Nine biopolymers mixture solutions ($R_{GA/Ch}^{\%Ch}$) were then prepared by mixing the appropriate amounts of both stock solutions, and by adding MilliQ-grade water when required, where *% Ch* indicates the total chitosan concentration and *GA/Ch* represents the weight ratios of gum Arabic to chitosan as follows: $R_3^{0.25}$, $R_5^{0.25}$, $R_7^{0.25}$, $R_3^{0.5}$, $R_5^{0.5}$, $R_7^{0.5}$, $R_3^{1.0}$, $R_5^{1.0}$ and $R_7^{1.0}$. The solutions were left to rest 72 h at room temperature prior to analysis. The upper soluble "equilibrium phases" were separated from the precipitated coacervate phases by decantation, and the yield of the coacervate phases were determined gravimetrically in dry basis (Espinosa-Andrews *et al.*, 2007).

2.3. Samples hydrolysis

Aliquots of 0.1-2.5 g/L of gum Arabic, 1 g/L chitosan, and 1.8 g of each of the different equilibrium phases were weighed out accurately at 10 g into tare 30 mL stopped Pyrex test tubes, and 4% w/w sulphuric acid was added to each tube. The tubes were placed in a mineral oil bath at 100 °C for 4 h and were then reweighed and made up to the own weight by addition of MilliQ-grade water. The solutions were then neutralized by adding 2.0 g $BaCO_3$ followed by shaking by 12 h (Randall *et al.*, 1989). The filtered hydrolyzates (0.45 μm) were analyzed by the HPLC system.

2.4. HPLC conditions

The sugar compositions of gum Arabic in the upper phase were determined following hydrolysis (Randall *et al.*, 1989) by HPLC (Agilent 1100 Series), equipped with a Rheodyne Model 7725i injector (Agilent, USA) fitted with a 20 μL loop. A Rezex RHM monosaccharide analysis column (7.8 mm ID x 300 mm, 8μm, Phenomenex, USA) and a refractive index detector at 35 °C (Agilent, 1100 Series, G1362A) were used. Samples were eluted using a water mobile phase at a flow rate of 0.2 mL/min and a temperature of 30 °C. The peaks were integrated with Agilent ChemStation software (Agilent, USA). The samples were carried out by triplicate.

2.5. Composition of gum Arabic in the equilibrium phase

D-galactose, L-arabinose, L-rhamnose, and D-glucuronic acid standards at concentrations of 0.05-10 g/L were injected into the HPLC. Each sugar retention time was determined from chromatograms, and standard curves for each sugar standard were obtained. Afterwards, different known concentrations (0.1-2.5 g/L) of the hydrolyzates from gum Arabic were injected into the HPLC, and their areas quantified. A standard curve of gum Arabic concentration versus the combined individual sugars areas was obtained, and the gum Arabic concentration in the equilibrium phases was calculated from this standard curve. Chitosan concentrations in the equilibrium phases, chitosan and gum Arabic concentrations in the coacervate phases were estimated by mass balance.

2.6. Statistical analysis

All the experiments were done in triplicate and statistical analysis was carried out using the Tukey's test with a significance level set at 1 % with the statistical software package NCSS (2001, Kaysville, Utah).

3. Results and discussion

The chromatograms of the D-glucuronic acid, D-galactose, L-arabinose, and L-rhamnose standards showed characteristic peaks at 23.5, 31.5, 32.3, and 33.8 min of retention time, respectively (Fig. 1). The calibration curve constructed exhibited excellent linearity by plotting the variation of peak areas as a function of concentration; four injections of standard solutions were assayed. The correlation coefficients were higher than 0.99 and intercepts that were not significantly different from zero ($p \leq 0.01$) (Fig. 2) in all cases. These results indicate the appropriateness of the HPLC conditions established for the sugars quantification.

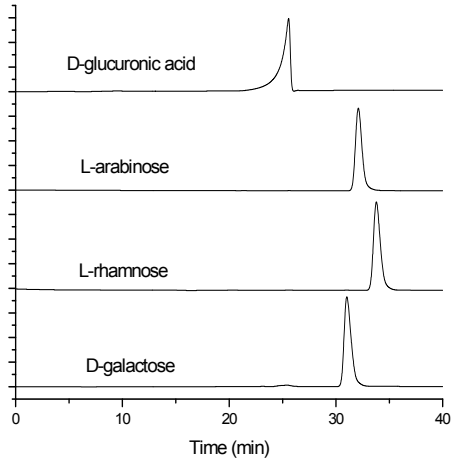

Fig. 1. HPLC chromatogram of D-glucuronic acid, D-galactose, L-arabinose, and L-rhamnose standards.

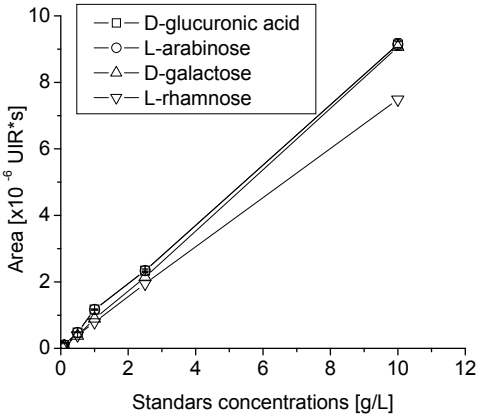

Fig. 2. Calibration curve of glucuronic acid, D-galactose, L-arabinose, and L-rhamnose standards.

Six hydrolyzed samples of gum Arabic at concentrations ranking between 0.1 to 2.5 g/L were injected into the HPLC, and the characteristic retention times for the different monosaccharides and the glucuronic acid were reproducible. However, the D-glucuronic acid data did not showed good linearity (R^2 = 0.89), compared to that exhibited by the

monosaccharides ($R^2 > 0.99$). The slightly upwards tilting curve obtained indicated that an overestimation of the D-glucuronic acid occurred, probably due to the production of artifacts arising from the interactions of the D-glucuronic acid with other species resulting from the hydrolysis. Thus, the calibration curve for gum Arabic was obtained from the combined monosaccharides areas (Fig. 3), and exhibited an excellent linearity ($R^2 = 0.99$).

The hydrolyzed chitosan sample was injected under the same conditions as the hydrolyzed gum Arabic samples into the HPLC did not produce chromatographic peaks. The functional group (counterion) adsorbed to the support's of the chromatograph column is responsible of the specific selectivities for each support. The counterion should have a lower selectivity for the functional group of chitosan, so that chitosan will displace the counterion and be adsorbed to the support. Chitosan properties, responsible for its interactions with a range of molecules, can make analytical methodology difficult, particularly creating stability problems in the chromatographic column, affecting efficiency and resolution of monosaccharide peaks (Estevinho *et al.*, 2008). However, this effect was minimized by periodic washing column. This result ensures that when hydrolyzing the equilibrium phases, the monosaccharides detected by the HPLC are sourced solely from gum Arabic. Chitosan samples must be hydrolyzed under more aggressive conditions (Boas, 1953; Novikov, 2004) or by enzymatic hydrolysis (Kuroiwa *et al.*, 2002; Il'ina *et al.*, 2004) for efficiently breaking down the molecule to its constituent monosaccharides.

Hydrolyzed samples of "equilibrium phase" of each biopolymers mixture solutions ($R^{\%Ch}_{GA/Ch}$) were injected in to the HPLC. Representative chromatograms of gum Arabic-chitosan equilibrium phases are shown in Fig. 4. The mass concentrations of gum Arabic in the equilibrium phases for each $R^{\%Ch}_{GA/Ch}$ were calculated from the calibration curve of gum Arabic (Fig. 3). Gum Arabic mass concentration in the coacervate phase was calculated subtracting the gum Arabic mass concentration calculated in the "equilibrium phase" by HPLC from the initial mass concentration of gum Arabic in the biopolymers mixture solution (Table 1).

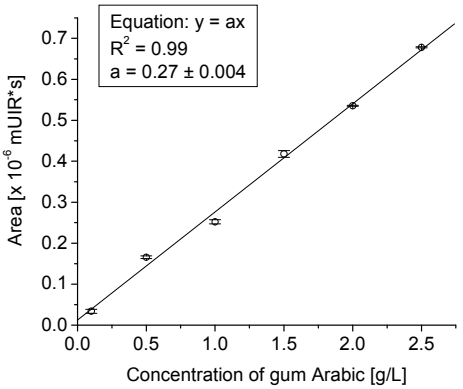

Fig. 3. Calibration curve of gum Arabic after acid hydrolysis.

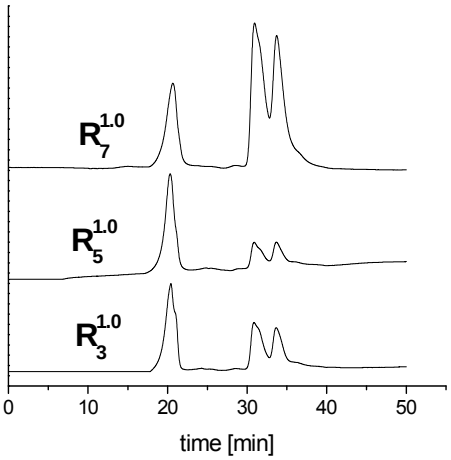

Fig. 4. Chitosan-gum Arabic complexes HPLC chromatograms.

These results were compared with those obtained by elemental analysis (Espinosa-Andrews *et al.,* 2007) for the same biopolymer mixtures used in this work. Data comparison obtained by both methods is shown in Fig. 5. There were non-significant differences ($p \leq 0.03$) between the methods, independently of the initial total biopolymers mixtures concentration used.

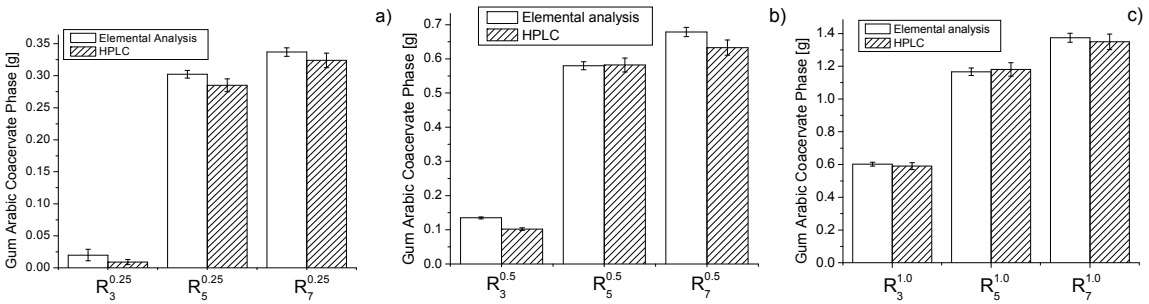

Fig. 5. Gum Arabic mass concentration calculated by HPLC and elemental analysis (Espinosa-Andrews *et al.*, 2007) for the different gum Arabic-chitosan solutions.

Table 1. Gum Arabic mass concentration in the coacervates phases and "equilibrium phases" for the different weight ratios.

Gum Arabic	$R_3^{0.25}$	$R_5^{0.25}$	$R_7^{0.25}$	$R_3^{0.5}$	$R_5^{0.5}$	$R_7^{0.5}$	$R_3^{1.0}$	$R_5^{1.0}$	$R_7^{1.0}$
Coacervate phase (g)	0.09	0.285	0.324	0.102	0.582	0.633	0.509	1.180	1.349
Equilibrium phase (g)	0.180	0.028	0.115	0.274	0.042	0.242	0.167	0.088	0.403

Conclusion

These results indicate that the gum Arabic mass concentration in binary gum Arabic-chitosan solutions can be readily and reliably quantified by analyzing the mixture hydrolyzates by HPLC.

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