Synthesis, characterization and functional properties of galactosylated derivatives of chitosan through amide formation

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ABSTRACT

Different low molecular weight chitosan (LMWC) and chitoooligosaccharide (COS) derivatives were obtained by the introduction of lactobionic acid (LA) through amide formation, obtaining different complexes COS-LA and LMWC-LA (1-5), with a degree of substitution (DS) between 3 and 16%. The synthesis of these derivatives was monitored by Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FT-IR), High-Performance Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC) and proton Nuclear Magnetic Resonance (^{1}H-NMR) analyses. Different functional properties, solubility, water binding capacity (WBC) and fat binding capacity (FBC), as well as the antioxidant activity (DPPH radical scavenging activity) of these derivatives were evaluated. Solubility, WBC and FBC increased in all of the chitosan derivatives respect to those of the native LMWC or COS. The most substituted chitosan derivative (LMWC-LA1, DS 15%) presented the highest value of solubility (14.4 mg mL^{-1}) while the highest levels of WBC and FBC were obtained for the derivative with a DS of 3% (LMWC-LA5; 4730% and 7100%, respectively). COS-LA showed a similar DPPH radical scavenging activity to that of COS in all concentrations tested (16.7-20.9% and 18.0-20.4%, respectively). An inverse relationship between the DS of chitosan derivatives and the antioxidant activity was observed. LMWC-LA5 (3% DS) was the chitosan derivative with the highest DPPH radical scavenging activity, being higher than LMWC in all the concentrations assayed (10.2-14.3% and 6.9-13.7%, respectively). Due to their enhanced functional properties, these chitosan derivatives could be considered as very promising for their future use as additives in the food industry (i.e. to bind fat and cholesterol or avoid hardening of foods).

Keywords: chitosan, chitooligosaccharides, glycosylation, lactobionic acid, functional properties

1. Introduction

In recent years, natural polymers have received more attention as an alternative to synthetic polymers in order to combine the production of manufactured products with the protection of environment, cost reductions and waste material recycling. Moreover, there is a trend focused in the exploitation of by-products and surpluses of food industry.

Chitin, a linear polymer of N-acetyl glucosamine units linked by β (1→4) bonds, is mainly obtained as a by product of the fishing industry. It is the second most abundant polymer in nature after cellulose and is the primary structural component of the shells of crustaceans, insects and fungal cell walls (Aranaz et al., 2009). Due to its low solubility, reactivity and low degradation rate is the major source of surface pollution in coastal areas (Kumar, 2000).

The deacetylated form of chitin is the chitosan, a polysaccharide composed of units of glucosamine (2-amino-2-deoxy-β-D-glucose) and N-acetyl glucosamine (2 acetamido-2-deoxy-D-glucose) linked by β (1→4) bonds. It is the only natural polysaccharide that presents cationic character due to free amino groups which play crucial roles in exhibiting various unique properties (Kurita, 2006). Some interesting properties include biocompatibility, biodegradability, non-toxicity and characteristic physicochemical and biological activities which have made it susceptible of multiple applications. The broad fields of application of chitosan include medicine, biotechnology, pharmaceutical, cosmetics, foods and agriculture (Aranaz et al., 2009; Prashanth, & Tharanathan, 2007).

However, despite this broad spectrum of properties, chitosan-related applications are limited by its insolubility at neutral or basic pH (Yang, Chou, & Li, 2002). Chitosan is soluble in acid aqueous solutions with pH values between 4.5 and 6.5 which constitutes a limitation on certain applications in food industry. In an attempt to improve the water solubility of chitosan, different strategies have been described. The introduction of hydrophilic groups by removing hydrogen atoms of free amino groups through different reactions provides chitosan modifications expanding its rate of solubilization. Thus acylation, alkylation and carboxymethylation, among other reactions, give derivatives with modified properties respect to those of native chitosan (Chung, Tsai, & Li, 2006; Sashiwa, & Shigemasa, 1999; Sreedhar, Aparna, Sairam, & Hebalkar, 2007).

Because of their high hydrophilicity and specificity, carbohydrates and derivatives have been used for chitosan modifications (Ying, Xiong, Wang, Sun, & Liu, 2011). Lactobionic acid (LA) (4-O-β-D-galactopyranosyl-D-gluconic acid) is a high value-added product obtained from lactose oxidation, with excellent properties for food and pharmaceutical applications. Besides, it presents different functional properties such as pH-reducing and strong mineral-complexing effect, which makes it suitable for diverse applications in food industry. LA can be used as acidulant with a sweet taste, as filler in cheese production, as firming agent and to fortify functional drinks with essential minerals such as Fe and Cu (Playne, & Crittenden, 2009; Toshiaki, Shuichi, Seiichiro, & Sakanori, 1995). Moreover, it has been described to be resistant to digestive enzymes being fermented by the intestinal flora, which makes it a potential prebiotic compound (Schaafsma, 2008).

Derivatives of chitosan with LA through amide formation give rise to branched derivatives with modified characteristics. These derivatives have been widely utilized as drug delivery systems for low molecular weight drugs, peptides and genes (Gao et al., 2003; Li, Li, Wang, & Qin, 2011; Zhang et al., 2011), as effective synthetic extracellular matrices for the attachment of hepatocytes (Chung et al., 2002; Mi et al., 2007; Park et al., 2003) and as stabilizer for obtaining iron oxide nanoparticles for the preparation of multifunction nanoprobes (Bahadur, Lee, Yoo, Choi, & Ghim, 2009). Moreover, different studies have been carried out in order to probe the non-toxicity of these products (Jain & Jain, 2010; Yang et al., 2010). However, there are few papers dealing with the functional and antioxidant properties of these chitosan derivatives, properties which are very interesting for the potential application of chitosan derivatives in the food industry.

Chitosan has been reported to possess several functional properties for its use in water and fat uptake, emulsification (Knorr, 1982), dye binding (Knorr, 1983) and gelation (Vorlop, & Klein, 1981). Chitosan has also been used as edible film or coating since it has been described to improve the storability of perishable foods by modifying the internal atmosphere as well as decreasing the transpiration losses (Nadarajah, Prinyawiwatkul, No, Sathivel, & Xu, 2006; Zhang, & Quantick, 1997). Moreover, the antimicrobial action against food spoilage microorganisms and the antioxidant properties of chitosan has been used for food preservation (Aranaz et al., 2009).

Chitosan modifications can also alter its functional properties; thus, the synthesis of new derivatives requires the study of these properties in order to evaluate if some changes occur respect to those of the native chitosan.

Therefore, the objective of this study was to obtain modified chitosans, through amide formation using lactobionic acid (LA), with improved functional properties comparing to that of native chitosan which can be of interest for food industry. Besides, because molecular weight has been described to affect functional and antioxidant properties of chitosan, this work has been performed using low molecular weight chitosan (LMWC) and chitooligosaccharides (COS).

2. Materials and methods

2.1 Materials

Lactobionic acid (LA) and glacial acetic acid were provided by Scharlau Chemie S.A. (Barcelona, Spain). Sodium acetate was from Panreac (Barcelona, Spain). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), N-ethyl-N'-[3-dimethylaminopropyl] carbodiimide (EDC), N-hydroxy succinimide (NHS), hydroxylamine hydrochloride and sodium 2-N-morpholinoethanesulfonate (MES) buffer were provided by Sigma-Aldrich Co (Steinheim, Germany). All reactive used were of analytical grade.

2.2 Samples

Low molecular weight chitosan (LMWC) was acquired from Sigma-Aldrich Co. (Steinheim, Germany).

Chitooligosaccharides (COS) were obtained by enzymatic hydrolysis of commercial LMWC using the commercial enzymatic preparation Pectinex Ultra SP-L produced by *Aspergillus aculeatus* (Novozymes, Dittingen, Switzerland) based in the methods of Cabrera & Van Cutsem (2005) and Wu (2011). Briefly, 10 g of LMWC were diluted in 500 mL of acetic acid (0.1 M), the pH was adjusted at 5.5 and incubated with 30 mL of the Pectinex Ultra SP-L solution (62 UE mL^{-1}) at 50 ºC for 16 hours. Reaction was stopped by heating at 100 ºC for 5 min; after, samples were

centrifuged at 17000 ×*g* for 10 min at 20 ºC. The supernatant was filtered and treated with 50% ethanol solution to separate the fraction of higher molecular weight. Finally, the supernatant obtained was treated with 75% ethanol and centrifuged at the same conditions to obtain a precipitate, which was recovered, washed with 75% ethanol and diluted in Milli-Q water and frozen for subsequent lyophilization.

2.3. Formation of low molecular weight chitosan-lactobionic acid (LMWC-LA) derivatives

LMWC-LA derivatives were obtained following the method described by Bahadur et al., (2009) using EDC and NHS as coupling agents. A volume of 10 mL of an aqueous solution of LA (150 g L^{-1}) was activated with 5 mL of NHS (180 g L^{-1}) and 5 mL of EDC (300 g L^{-1}) by stirring for 1 h. LMWC (1.0 g) were dissolved in 100 mL of 1.0% aqueous acetic acid by stirring for 24 h. The pH of the solution was adjusted to 6.5 by the slow addition of 0.1 M aqueous NaOH. Then, activated aqueous LA solution was dropped into LMWC solution under stirring and left for 72 h at room temperature. The resulting product was purified by dialysis (cut-off molecular weight of 12–14 kDa, Fisher Scientific) against Milli-Q water for 4 days, followed by lyophilization. In order to obtain derivatives with different degree of substitution, systems with different LMWC:LA ratios (w:w) (1:1.5, 1:1, 1:0.5, 1:0.25 and 1:0.125) (LMWC-LA1-LMWC-LA5) were prepared.

2.4. Formation of chitooligosaccharide-lactobionic acid (COS-LA) derivatives

COS were galactosylated using activated LA following the method described by Il'ina, & Varlamov (2007). Briefly, COS (0.5 g) were dissolved under stirring in 50 mL of 0.025 M MES buffer (pH 6.0) at room temperature. LA (0.40 g) was dissolved in 10 mL of 0.1 M MES buffer (pH 6.0) and supplemented with 0.49 g of NHS and 1.8 g of EDC. The activated LA was mixed with the COS solution and incubated at room temperature for 24 h under stirring. After incubation, the mixture was supplemented with 10 mM hydroxylamine hydrochloride; the pH of the reaction mixture was adjusted to 8.0 with 1 M NaOH. Then, galactosylated COS derivative was dialyzed against Milli-Q water for 96 h using Spectra/Por-3500 membranes (cut-off molecular weight of 3.5

kDa) (United States). The dialysate was centrifuged at 6000 ×*g* for 15 min to separate a slight precipitate formed in the course of dialysis and then lyophilized.

2.5. Analytical determinations

2.5.1. Sample characterization

Molar mass at the peak maximum (Mp) of LMWC, COS and their LA derivatives was determined by HPLC-Size Exclusion Chromatography (SEC). Two TSKGel columns (G2500PWXL × G5000PWXL) along with a PW_{XL} guard column (Tosoh Bioscience, Montgomeryville, PA, USA) were combined and coupled to a RID-10A Shimadzu refractive index (RI) detector. Analyses were performed at 25 ºC using 0.5 M acetic acid / 0.2 M sodium acetate as mobile phase with at a flow rate of 0.8 mL min^{-1}. Mobile phase was filtered before use through a HVLP filter with 0.45 μm pore size (Millipore, Irland). Samples were dissolved in a 1% acetic acid solution to obtain a concentration of 1 mg mL^{-1}, filtered through glass microfiber filters (3.1μm, 30mm) and 50 μL of chitosan solution were injected into the chromatographic system. Commercial pullulan samples of different molecular weights (0.3-800 kDa) were used for the calibration curve construction.

Data of **weight average molecular weight (Mw)**, **number average molecular weight (Mn)**, **intrinsic viscosity (IV)**, **hydrodynamic radius (Rh)** and **structural differences between samples** were obtained from HPLC analysis of corresponding samples using a Viscotek TDAmax system (Malvern Instruments, Worcestershire, UK) with refractive index (RI), light scattering and intrinsic viscosity detectors. Separation of samples was performed in a ViscoGEL C-MBMMW and two C-MBHMW columns. Elution was in isocratic using 5% acetic acid in water as mobile phase at a flow rate of 1 mL min^{-1}. Samples were dissolved in the mobile phase at approximately 0.7 mg mL^{-1} and filtered through a 0.45 μm nylon filter prior to being injected. The injection volume was 100 μL. OmniSEC software (Malvern Instruments, Worcestershire, UK) was used for acquisition and analysis of data.

The **degree of deacetylation (DD)** of the samples was determined by Fourier Transform Infrared Spectroscopy (FT-IR) and ^{1}H-NMR. FT-IR spectra were recorded in the middle infrared

(4000 cm^{-1} to 400 cm^{-1}) with a resolution of 4 cm^{-1} for 15 accumulations at room temperature in a Perkin-Elmer spectrometer (Spectrum one). Samples were prepared by grinding the dry LMWC or COS samples with KBr in a ratio 1:100 (1 mg sample:100 mg KBr) and then compressed to form discs. First, the degree of acetylation (DA) was calculated using the baselines and the equation proposed by Moore & Roberts (1980):

$$DA\ (\%) = ((A_{1655}/A_{3450})\times 100)/1.33 \quad (1)$$

where A_{1655} is the intensity of the absorption band of amide I, used as specific band for N-acetylation and A_{3450} is the intensity of the hydroxyl group absorption band, used as reference band; 1.33 is the ratio of the absorbance at 1655 cm^{-1} to that of the absorbance at 3450 cm^{-1} for fully N-acetylated chitosan. DD was then calculated using the following equation:

$$DD\ (\%) = 100 - DA\ (\%) \quad (2)$$

^{1}H-NMR analyses were performed following the method described by Hirai, Odani, & Nakajima (1991) on a Bruker DRX-500 spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) which operated at 500 MHz. Samples were dissolved in 2% DCl/D_2O solution to give a polymer concentration of 5 mg mL^{-1}. Measurements were carried out at 70 ºC. First, DA was calculated using the following equation:

$$DA\ (\%) = [(1/3 \times I_{CH3})/(1/6 \times I_{(H2\text{-}H6)})] \times 100 \quad (3)$$

where $I_{(H2\text{-}H6)}$ is the sum of integrals of H_2, H_3, H_4, H_5 and H_6 and I_{CH3} is the integral of the signals corresponding to the hydrogens of N-acetylglucosamine residues. DD was then calculated using the equation (2).

The **degree of substitution** (DS) of primary amino groups with LA residues in the LMWC and COS samples was calculated as the difference of the degree of deacetylation before and after reaction.

The **morphology** of LMWC, COS and their LA derivatives was studied by Scanning electron microscopy (SEM). Samples were coated with gold/palladium (80/60) and SEM images were obtained with a Philips XL30 ESEM microscope (Holland). Photographs were obtained at 50×, 100×, 500×, 2000× and 20000 × magnification.

2.5.2. Functional properties

The **solubility** was determined following the method described by Yang, et al. (2002) by dissolving 25 mg of LMWC, COS, LMWC-LA1-5, and COS-LA derivatives in 1mL of distilled water. The pH of the solutions was adjusted to 7 by adding drop-wise a solution of sodium hydroxide (2 M) and once pH of the solutions became stable, they were kept at 25ºC for 30 min. Samples were then centrifuged and the supernatant was lyophilized and weighed. Solubility was expressed as the weight of lyophilized supernatant per mL of distilled water.

Water binding capacity (WBC) and **fat binding capacity (FBC)** of LMWC, COS and their LA derivatives were measured using a modified method of Wang, & Kinsella (1976). Water or fat absorption measurements were carried out using weighed centrifuge tubes with 0.5 g sample and 10 mL of water or soybean oil. Mixture was shaken for 1 min to disperse the sample, left at ambient temperature for 30 min with shaking for 5 s every 10 min and centrifuged at 1220 ×*g* for 25 min. Then, the supernatant was decanted and discarded and the tube containing the precipitate was weighed again. WBC and FBC were calculated as follows:

$$\text{WBC (\%)} = [\text{bound water (g)}/\text{sample weight (g)}] \times 100 \quad (4)$$

$$\text{FBC (\%)} = [\text{bound fat (g)}/\text{sample weight (g)}] \times 100 \quad (5)$$

2.5.3. Evaluation of antioxidant activity

The **DPPH radical scavenging activity** of the samples was measured using the modified method of Blois (1958) described by Youn, No, & Prinyawiwatkul (2009). Briefly, 0.4 mL of solutions of COS or LMWC and their LA derivatives at different concentrations (0, 0.1, 0.2, 0.5, 1, 3 and 5 mg mL^{-1} in 1% acetic acid) were added to 3 mL of 0.1 mmol L^{-1} DPPH radical methanolic solution. The reaction mixture was shaken vigorously, stored in the dark at room temperature for 30 min, and the absorbance (A) was measured at 517 nm using a PowerWave^{TM} XS Microplate spectrophotometer (BioTek). All the assays were performed in duplicate. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation:

$$\text{Scavenging effect (\%)} = [(1 - A_{sample})/A_{control}] \times 100\% \quad (6)$$

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3. Results and discussion

3.1. LMWC and COS characterization

Before galactosylation reactions using LA (see Scheme 1), characterization of the commercial LMWC and purified COS fraction was carried out. Figure 1 shows the HPLC-SEC profiles obtained from COS (A) and LMWC (B) analysis. In the case of COS, a major peak with Mp of 9.5 kDa (peak 1 in Figure 1A) and two other peaks of 1.6 kDa and 0.3 kDa were observed (peaks 2 and 3, respectively), the retention time of the latter being similar to that of chitobiose standard. A single peak was obtained for LMWC (peak 1 in Figure 1B), showing an Mp of 150.7 kDa.

Also, structure of raw materials was studied by SEM. Figure 2 (A and C) shows the images obtained for COS and LMWC. As it can be observed, original LMWC (**C**), which was analyzed directly in powder form without any treatment, presented a very different structure comparing to the native COS (**A**). The final lyophilization step necessary for COS production has been described to affect the polymer structure, retaining most of the volume of the original hydrogel (García-Bermejo et al., 2012; Valentin, Bonell, Garrone, Renzo, & Quignard, 2007). In order to compare their structures, LMWC was solubilized in 1% acetic acid solution and submitted to lyophilization process (**D** in Figure 2). A similar laminar structure with filaments linked to each other was observed for both lyophilized COS and LMWC; however, the latter presented a smaller and closer pore structure due to its high molecular weight.

DD values obtained for LMWC and COS by ^{1}H-NMR were of 77% and 71%, respectively. Similar values (73% and 72%), were obtained when analyses were carried out by FT-IR. As it was observed by Il'ina & Varlamov (2007), the DD of COS only slightly changed respect to native chitosan during enzymatic hydrolysis and isolation.

3.2. LMWC-LA and COS-LA derivatives characterization

Different analytical determinations were performed in order to confirm the successful introduction of LA chains in both substrates, COS and LMWC, (see Scheme 1).

3.2.1. HPLC-SEC analysis

In order to check the formation of COS-LA and LMWC-LA derivatives, differences in molecular weight distribution respect to COS and LMWC samples were studied (Figure 1). COS-LA presented a similar HPLC profile to that of COS; however, a shift in the retention times occurred (peaks 1', 2' and 3' in Figure 1A) corresponding to a molecular weight of 15.2, 2.7 and 0.4 kDa respectively. The increment of molecular weight respect to that of original COS (see section 3.1) is an indicative of the satisfactory introduction of LA in COS chains.

However, similar chromatographic (HPLC-SEC) profiles were obtained for LMWC and its LMWC-LA derivative (peaks 1 and 1' in Figure 2B). In order to confirm the formation of complex between LMWC and LA, HPLC-SEC analyses using a triple detection (refractive index, light scattering and intrinsic viscosity) were also performed. These detectors give information of molecular weight, polydispersity, intrinsic viscosity and hydrodynamic volume of chitosan samples which provides a convenient and rapid way to characterize chitosans. For instance, in Table1 the different parameters found in LMWC-LA1 derivatives respect to native LMWC are included. The weight average molecular weight (Mw) and number average molecular weight (Mn) were higher in the chitosan derivative compared to LMWC, indicating an increase of weight due to the introduction of LA. The ratio of these two values (Mw/Mn), used to determine the polydispersity of the samples, was very similar in both samples, showing a medium molecular weight distribution, as expected for this type of sample (a natural biopolymer). The intrinsic viscosity (IV) can be used in combination with molecular weight to predict the structure of the molecule. In the synthesis of the derivatives, the positive charges of the amino groups of chitosan are diminished by the introduction of lactobionic acid through amide bond, which implies lower electrostatics repulsions between the chains, increasing the compaction of the molecules and thus increasing their density. This implies that they interact less with other molecules surrounding producing a less viscous solution. This agrees with IV and hydrodynamic size (Rh) data obtained for LMWC and LMWC-LA1; a decrease in both properties could be observed indicating a more compact structure for LMWC-LA derivative, probably due to the introduction of LA chains in the polymer. The same effect was

observed by Il'ina & Varlamov (2007) in the intrinsic viscosity of galactosylated chitosans obtained from LA with different degree of substitution.

3.2.2. SEM analysis

Structural changes on LMWC and COS produced by the introduction of LA chains were also studied by SEM. Figure 2 shows the images obtained for COS-LA (**B**) and LMWC-LA (**E**) derivatives. As it can be observed, some differences could be detected in their structure when comparing to their precursors (Figure **2A** and **2D**). A higher amount of binding fibers were observed between the laminar structures in both COS-LA and LMWC-LA derivatives, which could be an indicator of a structural change.

3.2.3. FT-IR analysis

Characteristic absorption bands at 3480-3440, 3260-3270 and 2960-2878 cm^{-1} corresponding to OH, NH and CH stretching regions of chitosan were observed in all samples (data not shown). Figure 3 shows the FT-IR spectra obtained in the region between 2000 and 400 cm^{-1} for LA, COS, LMWC, and their corresponding LA derivatives**.** Similar absorption bands were observed in LMWC (D) and COS (B) spectra. The bands at around of 1640, 1560 and 1380 cm^{-1} were assigned to amide I, II and III, respectively while the characteristic bands of saccharide structure appeared at around 1155 cm^{-1} (anti-symmetric stretching of the C-O-C bridge), 1080 and 1034 cm^{-1} (skeletal vibrations involving the C-O stretching) (Kassai, 2008). LA spectrum (Figure 3A) was characterized by an intense band at 1740 cm^{-1}, typical of C=O stretching of aldehyde groups.

Some differences could be observed between COS and the derivative COS-LA spectra (Figure 3B and 3C, respectively). A shift in the bands corresponding to amide I and II as well as an increase of their intensity comparing to the bands of saccharide chains could indicate the formation of new amide bonds and therefore, the successful interaction between the carboxylic group of LA and the amine groups of COS.

Figure 3 also shows the different spectra obtained for LMWC (D) and two LMWC-LA derivatives with different DS, LMWC-LA3 (E) and LMWC-LA1 (F). A similar behaviour to COS

was observed for LMWC derivatives. As it can be observed, the intensity of amide I and II bands increased when the introduction of LA in the chitosan chains occurred, being LMWC-LA1 a more substituted derivative (15% DS) than LMWC-LA3 (6% DS). Moreover, the band at 1603 cm^{-1} observed in LMWC corresponding to primary amino groups diminished in the derivatives as long as the glycosylation reaction occurred. These changes indicated the successful linkage of LA to chitosan chains during the reaction.

3.2.4. ^{1}H-NMR analysis

A typical ^{1}HNMR spectrum of COS and LMWC is shown in Figure 4A and 4B, respectively. The signal observed at δ 2.0 ppm was assigned to the hydrogens of the methyl moieties of the acetamide groups while the signal at δ 3.2 ppm was attributed to hydrogen bonded to the C2 of the glucosamine ring. The multiplet at δ 3.3-4.0 ppm was assigned to the hydrogens on the carbon atoms C3, C4, C5 and C6 and the signal at δ 4.9 ppm to the hydrogen on the C1 (Hirai, Odani & Nakajima, 1991; Park et al., 2003; Zhang et al., 2005; Mi et al., 2007).

Comparing the spectra, differences were observed between the native COS and LMWC and their LA derivatives. Signals were assigned following that reported in the bibliography (Hirai, Odani & Nakajima, 1991; Park et al., 2003; Zhang et al., 2005; Mi et al., 2007). As it can be observed in Figure 4C and 4D, new signals appeared in the range of 4.0-4.2 ppm due to the hydrogens bonded to the introduced galactose residues (Signal ***a*** in Figure 4C and 4D) and the chemical shift of CHCONH (4.5 ppm) (Signal ***1'*** in Figure 4C and 4D) was due to the reaction of the amine group on chitosan and the carboxylic acid on LA in the formation of the amide bond (Mi et al., 2007, Park et al., 2003; Zhang et al., 2005). This suggests again that the LA was successfully introduced to the chitosan backbone.

3.3. Functional properties

As it can be observed in Table 2, different degrees of substitution (3-15%) of LMWC derivatives were reached by varying the ratio between LMWC and LA. In the case of COS, the degree of substitution obtained was 16%. Since LA residues were linked at the primary amino

357 groups, responsible for characteristic properties of chitosan, a study of some of these properties of
358 modified chitosan was carried out.
359
360 *3.3.1. Solubility*
361 As seen from Table 2, the low solubility in water at pH 7 detected for native chitosan
362 (LMWC) increased in all of synthesized derivatives (LMWC-LA5-1), being higher when the DS
363 increased (3-15%) due to the introduction of hydrophilic LA, thus LMWC-LA1 derivative (DS
364 15%) presented the higher solubility value (14.37 mg/mL). The improvement of chitosan solubility
365 is very interesting since many of its biological and technological applications are limited by its
366 poor solubility at pHs above 6.5 (Aranaz et al., 2009). At higher pH, precipitation or gelation tends
367 to occur and the chitosan solution forms poly-ion complex with anionic hydrocolloids resulting in
368 gel formation (Kurita, 1998). On the other hand, it is important to remark that physicochemical and
369 biochemical activities of chitosan are due to the primary amino groups in the fundamental skeleton
370 and a significant modification of D-glucosamine units in chitosan is undesirable (Park et al., 2003).
371 It has been described, that a substitution of 3-20% of amino groups of chitosan with LA is enough
372 to enhance its solubility, the remaining amount of amino groups (74-62% in our derivatives) being
373 sufficient to ensure the biological activity of polymer (Il'ina, & Varlamov, 2007).
374 COS, which was readily soluble in water (pH 7), experimented an increase in its solubility
375 with the introduction of LA, ranging from 148.6 mg/mL to 536.60 mg/mL in COS-LA derivative.
376
377 *3.3.2. Water binding capacity (WBC) and fat binding capacity (FBC)*
378 WBC and FBC values of LMWC, COS and their corresponding LA derivatives are shown
379 in Table 2. WBC and FBC values obtained for LMWC were in the range described by Cho, No, &
380 Meyers (1998), No, Lee, & Meyers, (2000) and Rout (2001) for different commercial chitosan
381 products. As it can be observed, an improvement in WBC and FBC was also detected for LMWC
382 and COS derivatives respect to the native samples.
383 WBC values differed between LMWC and its LA derivatives, ranging from 1711 to
384 4703%. It must be pointed out that those values were much higher than that of original LMWC

(454%) and also than the values reported by Cho et al., (1998), No et al., (2000) and Rout (2001) for different commercial chitosans (458-805%, 355-611% and 581-1150%, respectively). The samples that presented the highest values of WBC were LMWC-LA4 and 5 which presented lower DS (4 and 3%, respectively). WBC of COS and its derivative could not be measured since they were already soluble under the experimental conditions. High values of WBC have also been described for other types of chitosan derivatives. Bidgoli, Zamani, & Taherzadeh, (2010) and Wu, Black, Santacana-Laffitte, & Patrick, (2007) obtained values of WBC of 11700 and 4000 % for carboxymethyl and glutaraldehyde collagen chitosan derivatives, respectively, the former being considered superabsorbent.

FBC of LMWC derivatives were significantly higher than that observed for the native chitosan, ranging from 5313 to 7100% (Table 2). Unlike that observed for WBC, this functional property was found to be related to DS of the samples. Inversely to solubility, it can be seen that the greater DS, the smaller FBC of chitosan derivatives. It has been described that FBC of chitosan increase with the increase of both DD and molecular weight (Mw) (Xia, Liu, Zhang, & Chen, 2011; Zhang et al., 2010). Molecules with higher DD possess more free amino groups which can interact via electrostatic attraction with anionic substances such as fatty acids and bile acids. When the DD and the particle size are comparable, the FBC of chitosan is enhanced with increasing Mw, suggesting that during the fat-binding process, the fat molecules are embedded in the long chain of chitosan; hence, a larger molecular weight with longer chains implies more fats that can be embedded (Zhang et al., 2010). This effect could be observed when comparing samples LMWC-LA1 and COS-LA, both with a similar DS and DD but with different molecular weight, which presented FBC of 5313% and 4744%, respectively.

As occurred for WBC, the values obtained for FBC for the LMWC-LA derivatives were higher than those reported for commercial chitosans (Cho et al., 1998; No et al., 2000; Ocloo, Quayson, Serfor-Armah, & Woode, 2011; Rout, 2001), which varied between 314-535%, 217-403%, 587-706% and 431-561%, respectively. This suggests that the introduction of LA in chitosan chains enhance its FBC, which may be of practical importance, since it may allow

412 reducing the daily dose of chitosan to obtain the same dietary effect (Czechowska-Biskup, Rokita,
413 Ulanski, & Rosiak, 2005).

414 In summary, due to the promising values obtained for FBC of the different complexes,
415 COS-LA and LMWC-LA could be used as dietary ingredients to reduce the absorption of fat and
416 cholesterol in the diet and therefore to be used in obesity control (Aranaz et al., (2009)). On the
417 other hand, due to the high values of WBC obtained for LMWC derivates, these complexes could
418 be used to avoid hardening of foods such as bread, biscuits or dough. The increased solubility of
419 the derivatives respect to their precursors amplifies their applications in the food industry and
420 allows easier handling when using as additives.

421 It must be pointed out that, to the best of our knowledge, this is the first time that WBC and
422 FBC values for LMWC-LA and COS-LA derivatives are reported.

423

424 *3.3.3. Antioxidant properties*

425 DPPH scavenging effect of LMWC, COS and their LA derivatives is plotted in Figure 5A
426 and 5B for the different concentrations tested. COS, with a lower molecular weight than LMWC,
427 presented a higher antioxidant activity, which is in accordance with that reported by other authors
428 (Chien, Sheu, Huang, & Su, 2007; Cho, No, & Prinyawiwatkul, 2008; Kim, & Thomas, 2007;
429 Tomida et al., 2009). The latter studied the effect of molecular weight on antioxidants properties of
430 chitosans. They described that high molecular weight leads to more compact structures with lower
431 mobility, increasing the possibility of inter- and intramolecular bonding. This effect decreases the
432 reactivity of hydroxyl and amino groups as the chance of exposure of these groups can be restricted
433 and thus, antioxidant capacity is diminished.

434 Regarding LA derivatives, it was observed that COS-LA derivative presented a similar
435 antioxidant activity to its precursor (Figure 5A). The mechanism of radical scavenging activity of
436 COS or LMWC is not clear; it is attributed that amino and hydroxyl groups attached to C-2, C-3
437 and C-6 positions of the pyranose ring react with unstable free radicals to form stable
438 macromolecule radicals (Huang, Mendis, & Kim, 2005). The introduction of LA chains reduces the
439 number of free amino groups of the molecule, affecting the scavenging activity of these groups.

However, in the derivatives, this effect could be in part offset by an increment of the hydroxyl groups attached to C-2, C-3 and C-6 positions of the pyranose ring of LA.

As Figure 5B shows, an inverse relationship between the DS of LMWC-LA derivatives and the DPPH scavenging activity was also observed at low concentration up to 1 mg/mL. Thus, the LMWC-LA5 derivative with the lower DS (3%) presented the higher antioxidant activity. This effect could be conditioned by two parameters: i) increase of molecular weight; as LA chains are introduced in the chitosan backbone an increment in the molecular weight is occurring; ii) reduction of the number of free amino groups and thus, reduction of abstractable hydrogen atoms of the molecule, affecting the scavenging activity of these groups. The same behaviour has also been described for chitosan derivatives with different DS by other authors (Huang, Rajapakse, &, Kim, 2006; Lin, & Chou, 2004).

4. Conclusions

In this work, different chitosan- (LMWC) and chitooligosaccharides (COS) lactobionic acid (LA) derivatives were synthesized and thoroughly characterized. The improvement of different functional properties of the synthesized derivatives respect to those of native LMWC or COS has also been demonstrated. Among the different derivatives obtained, LMWC-LA5, presented very high values of WBC and FBC, much higher than its control, which, together with an improved solubility and antioxidant activity, makes this derivative a very promising compound to be applied as an additive in the food industry.

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Figure captions

Scheme 1. Synthesis scheme of chitosan-lactobionic acid derivatives. Letters refer to the different signal assignments of ^{1}H-NRM spectra (Figure 4)

Figure 1.- HPLC SEC–RI profiles of **(A)** chitooligosaccharides (COS) (peaks 1, 2 and 3) and chitooligosaccharide:lactobionic acid derivative (COS-LA) (peaks 1', 2' and 3') and **(B)** low molecular weight chitosan (LMWC) (peak 1) and low molecular weight chitosan:lactobionic acid derivative (LMWC-LA1) (peak 1').

Figure 2.- SEM images obtained for chitooligosaccharides (COS) **(A);** commercial low molecular weight chitosan (LMWC) **(C**), and lyophilized commercial LMWC **(D)** and their respective lactobionic acid derivatives, (COS-LA (**B**) and LMWC-LA (**E**)).

Figure 3.- FT-IR profiles obtained for lactobionic acid (LA) **(A)**, chitooligosaccharides (COS) **(B)**, chitooligosaccharide:lactobionic acid derivative (COS-LA) **(C)**; low molecular weight chitosan (LMWC) **(D);** low molecular weight chitosan:lactobionic acid derivative 3 (LMWC-LA3) (Degree of substitution DS 6%) **(E)**; and low molecular weight chitosan:lactobionic acid derivative 1 (LMWC-LA1 DS 15%) **(F)**.

Figure 4.- ^{1}H-RMN spectrum of **(A)** chitooligosaccharides (COS), **(B)** low molecular weight chitosan (LMWC), **(C)** chitooligosaccharide:lactobionic acid derivative (COS-LA), **(D)** and low molecular weight chitosan:lactobionic acid derivative 3 (LMWC-LA 3 DS 6%). HOD: Signal corresponding to solvent. For letters assignments, see Scheme 1.

Figure 5.- % of DPPH radical scavenging activity (%) of chitooligosaccharides (COS) **(A)** and low molecular weight chitosan (LMWC) **(B)** and their lactobionic acid (LA) derivatives (LMWC-LA1-5) after 30 min of reaction.

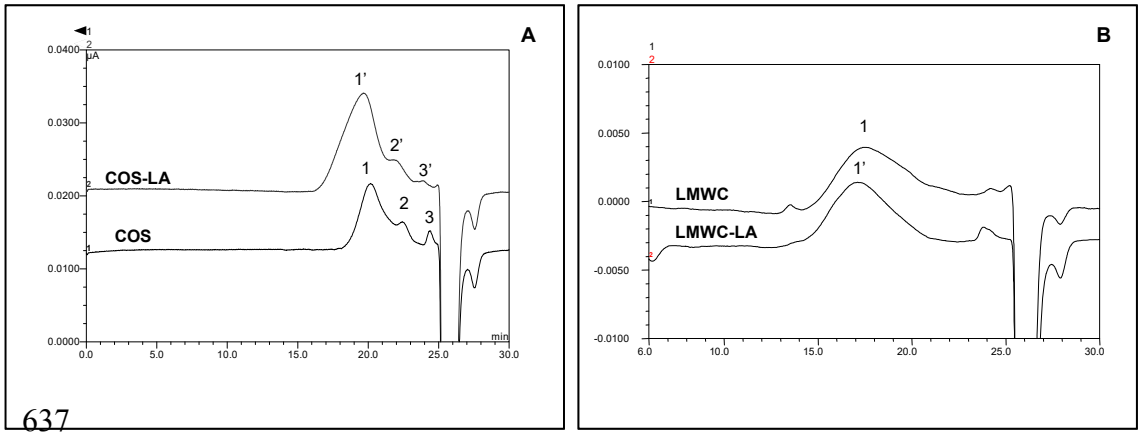
Table 1.- Quantitative results obtained from analysis of low molecular weight chitosan (LMWC) and LMWC-lactobionic acid (LA) (LMWC-LA1) derivative using a Viscotesk TDA max system with refractive index (RI), light scattering and intrinsic viscosity detectors. M_W: average molecular weight; Mn: number average molecular weight; Mw/Mn:polydispersity of the sample**;** IV intrinsic viscosity; Rh: hydrodynamic radius.

SAMPLES	M_W (kDa)	Mn (kDa)	Mw/Mn	IV (dL/g)	Rh (nm)
LMWC	76.2	36.0	2.1	7.8	19.1
LMWCLA1	102.2	53.6	1.9	4.2	17.2

Table 2.- Degree of substitution (DS) and functional properties of LMWC, COS and their lactobionic acid (LA) derivatives.

SAMPLES	Functional properties			
	DS (%)	Solubility (mg/mL)	WBC (%)	FBC (%)
LMWC	-	0.47±0.11	453.46±13.28	285.08±36.26
LMWCLA 1	15	14.37 ± 5.38	2448.91 ± 306.75	5313.22 ± 573.65
LMWCLA 2	10	13.88 ± 1.04	1841.63 ± 292.61	5584.56 ± 855.66
LMWCLA 3	6	10.13± 1.95	1711.44 ± 107.65	5742.25 ±716.61
LMWCLA 4	4	5.04 ± 1.33	3186.06 ± 61.65	6617.32 ± 406.12
LMWCLA 5	3	5.40 ± 0.88	4703.22 ± 347.55	7100.65 ±827.55
COS	-	148.6 ± 1.98	soluble	3345.99 ± 82.66
COSLA	16	536.60 ± 3.68	soluble	4744.06 ± 491.72

636 Figure 1.



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639 Figure 2.

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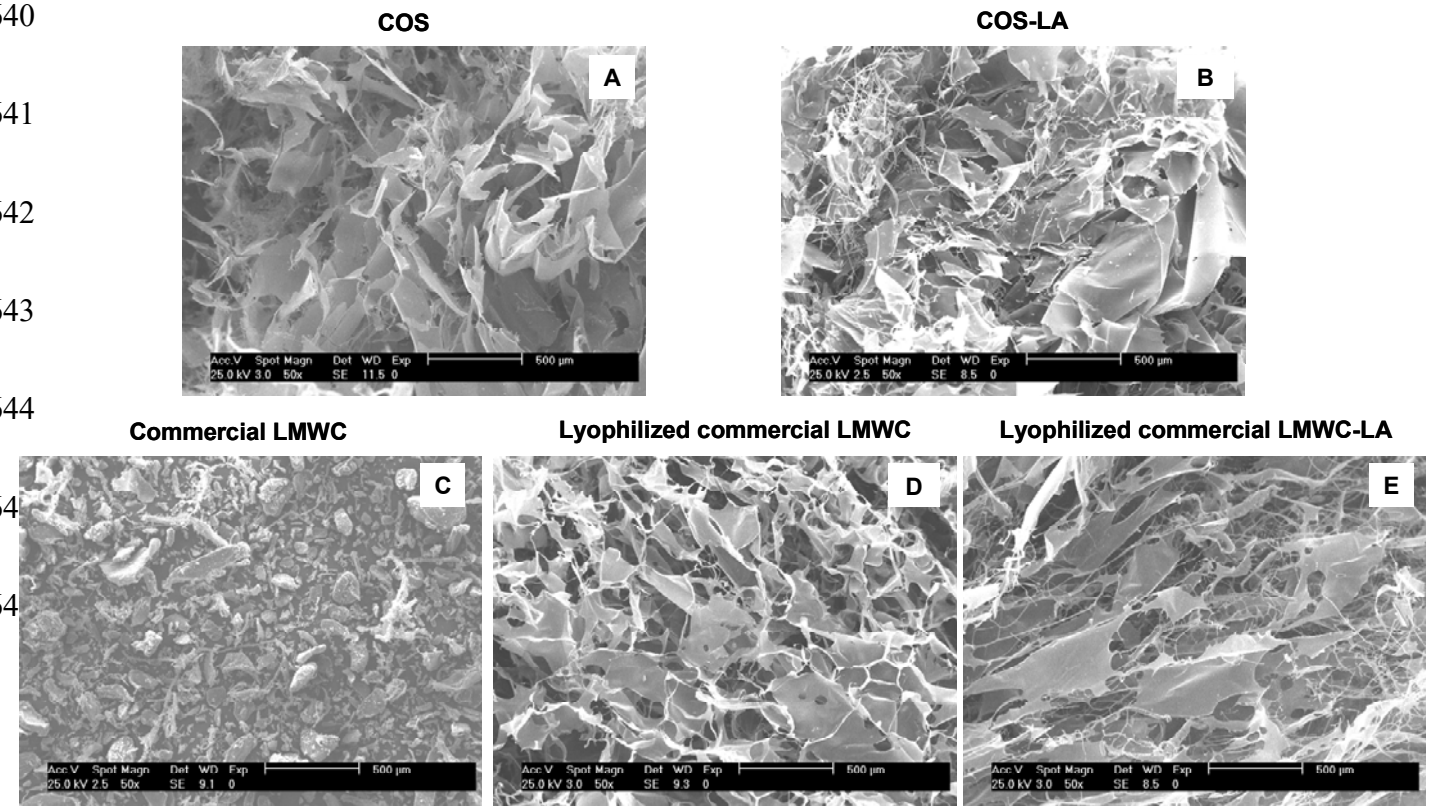

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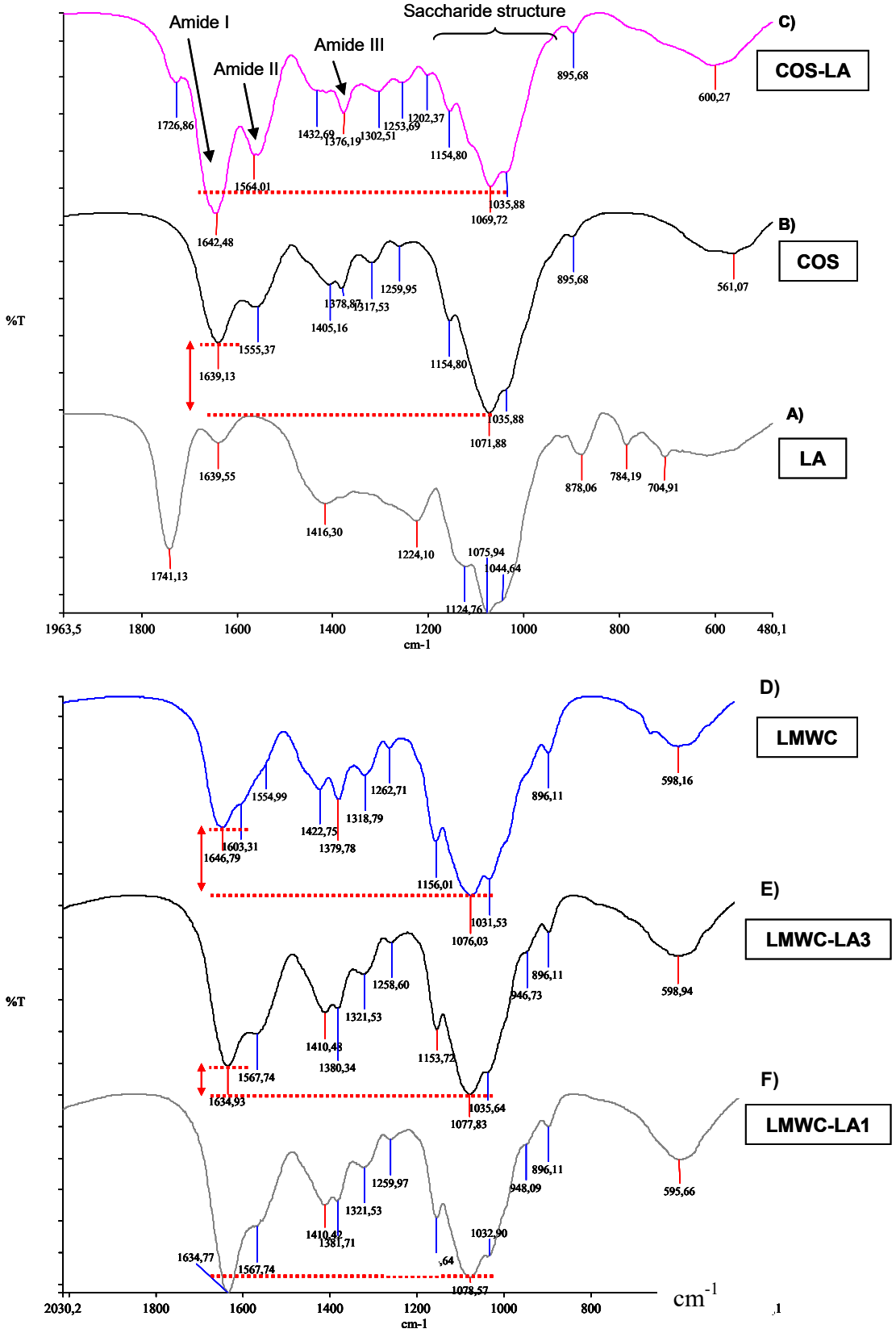
647 Figure3



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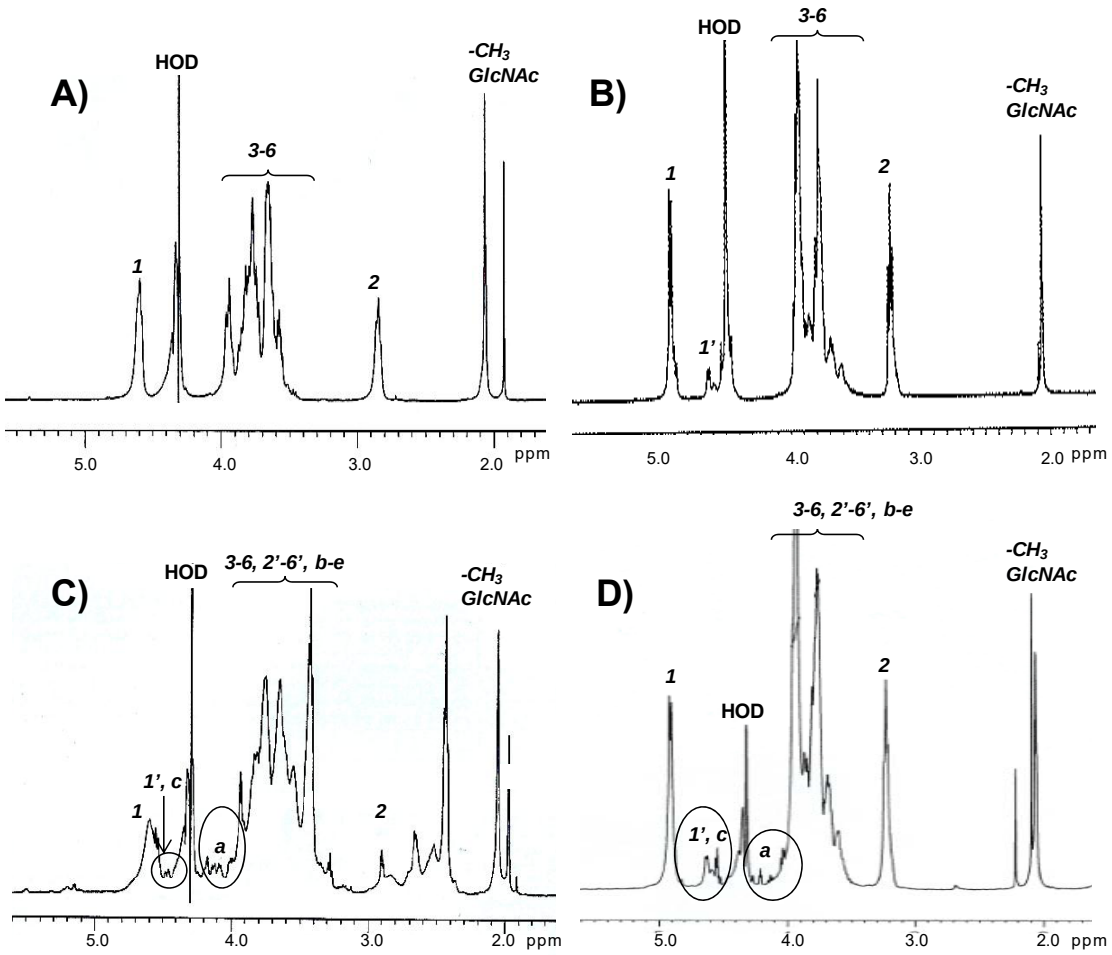

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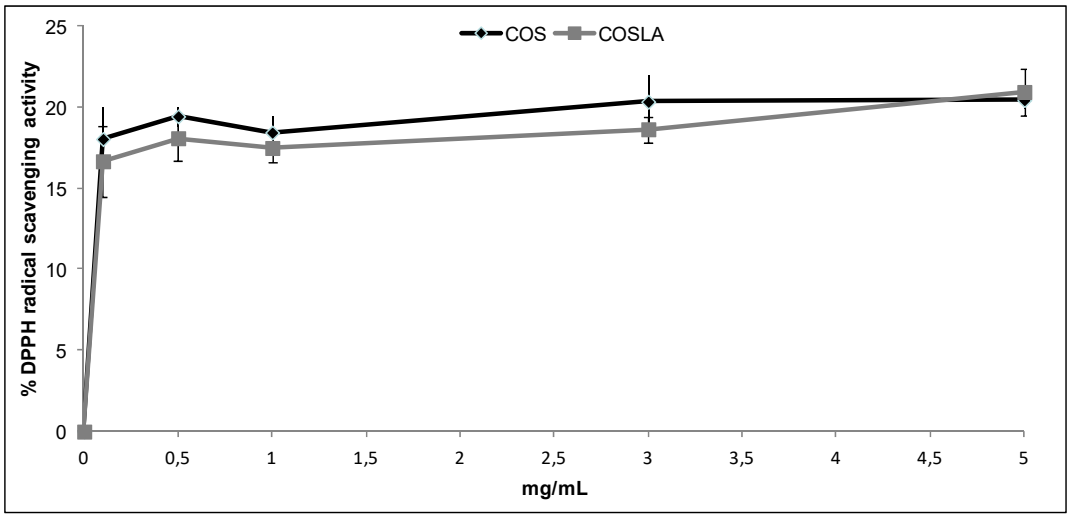
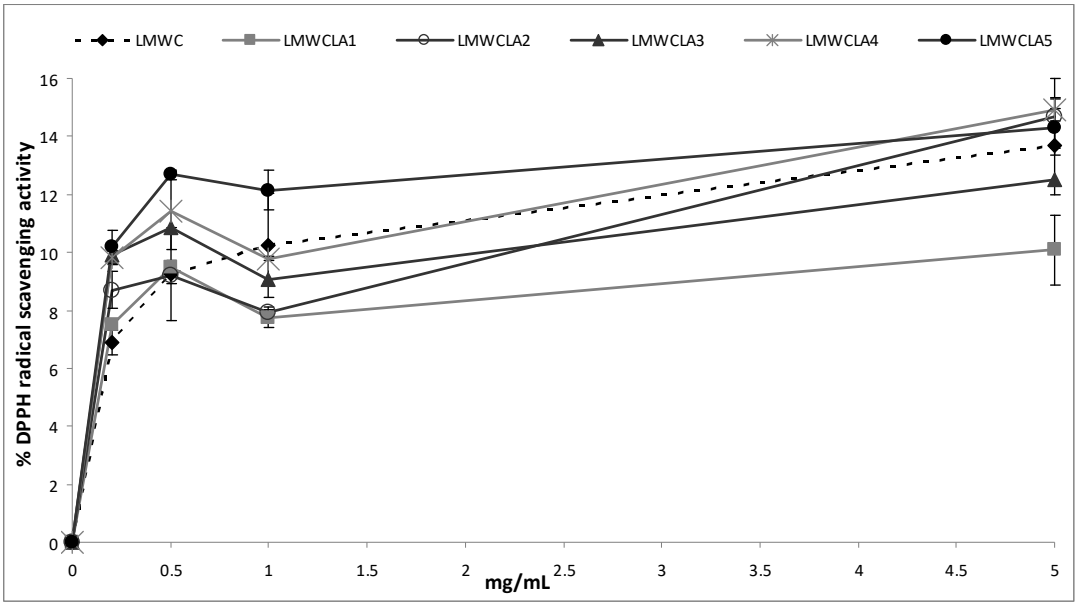
652 Figure 5.

A)



B)



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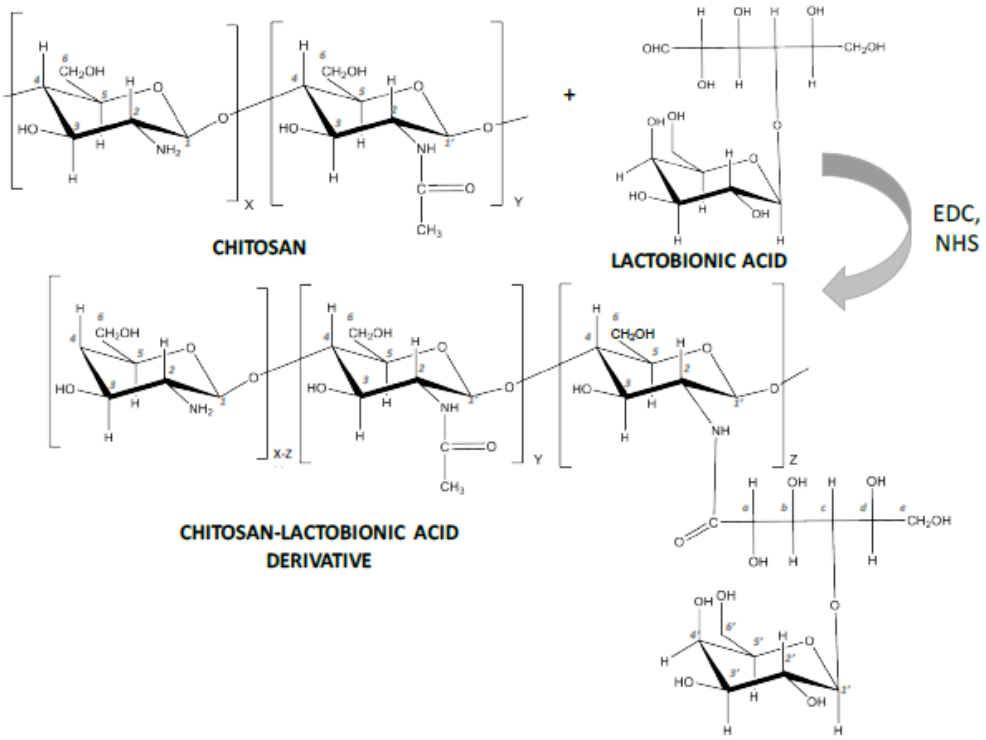
Scheme 1.



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