Supercritical CO_2 impregnation of lactulose on chitosan: A comparison between scaffolds and microspheres form.

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Abstract

Nowadays, the application of green chemistry principles in the production of new polymeric materials is receiving an increasing attention. In the present work, we have investigated the impregnation of chitosan with lactulose using supercritical fluids under various operating conditions, in order to improve the solubility of this natural polymer at neutral or basic pH. A comparison between chitosan scaffolds and microspheres is also presented; both chitosans were characterized using scanning electron microscopy (SEM), mercury intrusion porosimetry (MIP) and Fourier transform infrared spectroscopy (FTIR). The degree of impregnation was evaluated by quantitative gas chromatography (GC-FID) analysis and interactions chitosan-lactulose by ninhydrin method. The supercritical carbon dioxide impregnation proved to be feasible for both chitosan forms. The highest impregnation yield (8.6%) was obtained for chitosan scaffolds using the following impregnation parameters: continuous process, 60 minutes contact time, 14% (v/v) of co-

solvent ethanol:water (95:5), depressurization rate equal to 3.3 bar/min, 100 bar of pressure and 100°C. Under these conditions, Maillard reaction also occurred.

Keywords: Chitosan, Scaffolds, Microspheres, Supercritical CO_2, Impregnation, Lactulose

1. Introduction

Chitosan is a cationic polymer derived from chitin comprising monomers of glucosamine and N-acetyl glucosamine. Chitin is the second most abundant natural-origin polysaccharide after cellulose, found in the exoskeletons of arthropods. Chitosan is mainly obtained by deacetylation of chitin from crustacean shells (crabs, shrimp, lobsters…) because of the large quantity available as seafood industry wastes [1].

The degree of deacetylation and molecular weight of chitosan determines physicochemical properties and biological activities of chitosan [2]. Chitosan has been processed in several forms, namely, scaffolds and microspheres, by a variety of methods. Chitosan scaffolds can be prepared by freeze-drying of a chitosan gel solution [3] while microspheres can be obtained by drying gel beads of the natural polymer under supercritical CO_2 conditions; this particular method makes the accessibility of chitosan functional groups easy [4,5].

The physicochemical and biological properties of chitosan such as reactivity [6], biodegradation [7], antimicrobial [8], antioxidant [9], etc., along with the ability to be processed in different ways makes chitosan an excellent material with several applications in many fields, particularly in medicine and pharmacy, textile and paper industry, agriculture and biotechnology. Despite its high potential in the food processing as food additive or for nutraceutical encapsulation, its industrial utilization has not been

consolidated mainly due to the limited solubility of chitosan in neutral and basic solutions [10,11]. However, it is known that incorporation of 3–30% of mono- or disaccharide residues into the chitosan molecule changed the solubility of its derivatives at pH higher than the apparent acidity constant of chitosan amino groups (6.3–6.7) [12].

Lactulose (4-o-β-D-galactopyranosyl-D-fructose) is a synthetic ketose disaccharide obtained from lactose by alkaline isomerization [13]. Lactulose is a prebiotic carbohydrate with ability to stimulate the growth and activity of bifidobacteria and lactobacilli present in the gastrointestinal tract, performing many important functions such as protection from food-borne illnesses and allergies, regulating hormone balance, and enhancing immunity [14,15].

Supercritical fluids (SCFs) are considered an attractive alternative to organic solvents for polymer processing [16]. Besides its environmental friendly status, the main reason for using SCFs in polymer processing comes from the opportunity to utilize SCFs favorable properties such as high diffusivities, low viscosities, and near zero surface tension which allow a rapid penetration into a high variety of matrices. Although there is a wide range of compounds that can be used as supercritical fluids, carbon dioxide (SC-CO_2) is, by far, the most used due to its moderate critical temperature (31 ºC) and pressure (72 bar), its cheapness and its GRAS (generally recognized as safe) status by FDA (Food and Drug Administration) and EFSA (European Food Safety Authority). Another advantage is that CO_2 is gaseous at room temperature and pressure which provides solvent-free polymeric matrices.

Several researchers have developed methods to improve or modify the properties of chitosan based on the use of supercritical technology; for instance, in the last decades, supercritical fluids have been used to synthesize new chitosan derivatives [17,18] using

reductive sugars such as glucose or maltooligossacharides [19] or to carry out impregnation of chitosan for drug release control [20]. The use of a prebiotic sugar, such as lactulose, for chitosan modification has never been attempted although chemical and biological properties of the resultant chitosan would be greatly improved in terms of solubility and bioactivity. Therefore, in the present work, supercritical solvent impregnation (SSI) of chitosan with lactulose has been studied. It is important to distinguish between two mechanisms of impregnation assisted by supercritical fluids [16] that can either occur alone or simultaneously, depending of the impregnation conditions; the two mechanisms are the deposition of the target compound in the polymer matrix and the chemical interaction compound-chitosan.

Impregnation efficiency results from a complex mechanism that involves interactions between the solute (lactulose), the mobile phase (carbon dioxide + cosolvent) and the matrix (chitosan). The relative strength of all binary interactions will contribute to the final partitioning of the solute between the mobile phase and the matrix [3]. The phase behavior of different polymers, such as chitosan, in supercritical carbon dioxide has been widely studied in recent years [5,21]. It is also known from literature that solute solubility in CO_2 will increase when using a cosolvent with the same polar characteristics of the solute [22]. Undoubtedly, knowing the solubility of the solute in the supercritical media is crucial to optimize impregnation conditions. In this sense, solubility of lactulose in SC-CO_2 with (ethanol + water) as cosolvent at certain operational conditions (pressure and temperature) has been previously reported by Montañes et al., 2009 [23].

Thus, the main goal of this work was to study and optimize the impregnation of lactulose into two chitosan forms: chitosan scaffolds and chitosan microspheres. Supercritical fluid impregnation methodology has been used employing CO_2 and

ethanol:water mixtures to obtain a water-soluble chitosan that might find applications in the food industry as a functional ingredient.

2. Materials and methods

2.1. Materials

Two types of chitosan were purchased from Sigma-Aldrich (Madrid, Spain): a low molecular weight (150 kDa) and a medium molecular weight (350 kDa). Lactulose (98% purity), internal standard (phenyl-β-D-glucoside), methanol and derivatizing reagents (hydroxylamine hydrochloride, hexamethyldisilazane and trifluoroacetic acid) were also obtained from Sigma-Aldrich. Acetic acid and sodium hydroxide were purchased from Panreac (Barcelona, Spain), ethanol absolute was from Prolabo (Madrid, Spain), and pyridine was supplied by Merck (Darmstadt, Germany). Ultrapure water quality (18.2 MΩcm) with 1–5 ppb total organic carbon (TOC) and <0.001 EU/mL pyrogen levels was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica, USA). Carbon dioxide (CO_2) liquefied at high pressure used in supercritical fluid impregnation was supplied by Praxair (Madrid, Spain). Washed glass wool chemically pure was acquired from Panreac.

2.2. Preparation of lyophilized chitosan scaffolds

A solution of 1 wt% of chitosan (low molecular weight) in a diluted acetic acid solution (1 wt% in water) was prepared. Total dissolution was obtained by stirring during 5 h at room temperature. The solution was poured into cylindrical moulds, which were frozen first in liquid nitrogen and then at -80ºC. After this procedure the samples were lyophilized

using a freeze-dryer Labconco 79480 (Missouri, USA) for 4 days to completely remove the frozen solvent [3].

2.3. Preparation of $scCO_2$ dried chitosan microspheres

A solution of 1 wt% chitosan (medium molecular weight) in a diluted acetic acid solution (2 wt% in water) was prepared. Total dissolution was obtained by stirring during 5 h at room temperature. This solution was added dropwise into a sodium hydroxide solution (5 wt% in water) through a burette. The chitosan microspheres were repeatedly washed with ultrapure water until neutral pH, and then dehydrated by immersion in a series of successive ethanol–water baths of increasing alcohol concentration (10, 30, 50, 70, 90, and 100%) for 15 min each. Finally, the microspheres were dried under supercritical CO_2 conditions (74 bars, 32 ºC) during 2 h in the Suprex Prep Master apparatus described below [4].

2.4. Supercritical solvent impregnation (SSI) process

The supercritical impregnation apparatus used to perform the experiments is schematically presented in Figure 1. The equipment is based on a Suprex Prep Master (Suprex Corporation, Pittsburg, PA, USA) with several modifications. It has a thermostatic oven heated by air convection where the impregnation cell (with approximately 10 cm^3 of internal volume) containing the sample is placed. A pre-heater system was employed by placing a heating coil inside a glycerin bath (JP Selecta Agimatic N, JP Selecta S.A., Abrera, Spain) to guarantee that the fluid employed in all the experiments reaches the high pressure vessel at the target temperature. The system is also equipped with a Suprex solvent modifier pump. After the modifier pump, a check valve (Swagelok SS-CHS2-BU-10,

143 Swagelok Corporation, Solon, OH, USA) was used. Another Swagelok check valve and a
144 micrometering valve (Hoke SS-SS4-BU-VH, Hoke Incorporated, Spartanburg, SC, USA)
145 were placed after the impregnation cell to manually control the flow. A linear restrictor
146 consisting on a silica capillary (50 cm x 75µ i.d.) was used to control slow decompression
147 of the system. Carbon dioxide flow rate was measured by a computer-controlled mass flow
148 meter (EL-FLOW Mass Flow Meter/Controller F-111C, Bronkhorst High-Tech BV, AK
149 Ruurlo, The Netherlands).

150 The SSI method consists in introducing the compressed fluid (mixture of CO_2 and
151 cosolvent) into the impregnation cell for a predetermined time period (either 60 or 180
152 minutes fixed time or 3 loading cycles of 60 min each). The impregnation cell was
153 previously set at the desired operational conditions (T and P). At the end of this period, the
154 system was slowly depressurized. Impregnated chitosan samples were then recovered in a
155 semi-dry final state and stored at room temperature in a desiccator with silica gel.

156 SSI experiments were performed either in a batch or continuous mode in order to
157 evaluate the performance of these two techniques. The batch impregnation process was
158 carried out with the valves placed after the impregnation cell closed. The continuous mode
159 consisted of a 60 minutes of dynamic impregnation with the supercritical carbon dioxide
160 flow rate adjusted at 1.2 g/minute.

161 The cosolvent was selected based on previous results reporting the solubility of
162 lactulose in supercritical media [23] and was a mixture of ethanol:water (95:5 v/v). The
163 vessel was loaded with the selected amount of chitosan (500 mg or 300 mg) processed in
164 scaffolds or microspheres form and lactulose (50 mg or 150 mg) in a 10:1 or 2:1 ratio that
165 was modified depending on the experiment. Lactulose was placed on the bottom side of the

vessel, so that the supercritical fluid comes in contact first with lactulose and then with the polymeric matrix. Both were separated by a piece of glass wool in order to prevent contact between them and therefore, to avoid contamination of the surface of the chitosan. Lactulose was always in excess, what was verified visually by checking the residual lactulose in the impregnation vessel after the process.

The operating pressure and temperature and the amount of cosolvent for each experiment were established considering the solubility of lactulose (saturated environment) in the mixture of compressed fluid and cosolvent, and according to data reported previously [23].

2.5. Chitosan characterization procedures

2.5.1. Scanning electron microscopy (SEM)

The surface of polymer samples was analyzed and imaged by scanning electron microscopy (SEM, Philips, XL-30 model, Holland), after gold palladium coating, approximately 50 A°, in an argon atmosphere. Images were taken with an accelerating voltage of 25 kV at various levels of magnification.

2.5.2. Mercury Intrusion Porosimetry (MIP)

Porosity measurements (pore size, surface area, % porosity) were carried out in chitosan samples using a mercury intrusion porosimeter PoreMaster Series 60 model (Quantachrome Instruments, Boynton Beach, Florida, USA). The MIP was performed and analyzed under standard conditions (Hg surface tension σ = 480.00 erg/cm^2, Hg contact angle Θ = 140.00º, pressure range 0-50 PSIA for low pressure and 20-60000 PSIA for high pressure experiments).

2.5.3. Fourier transform infrared spectroscopy (FT-IR)

Infrared spectra were obtained with an FT-IR spectrometer (Perkin Elmer Spectrum One, California, USA) by using the KBr pellet method and were recorded by an average of 64 scans at a resolution of 4 cm^{-1}.

2.6. Quantitative gas chromatography (GC) analysis of lactulose loading

2.6.1. Sample preparation

The lactulose-loaded chitosans were weighed and immersed in Milli-Q water for 20 min with constant stirring in order to extract all the impregnated lactulose. Chitosan was precipitated at a pH between 7 and 8.5. One ml of the supernatant was mixed with 400 µl of phenyl-β-D-glucoside (internal standard) (0.5 mg/ml) and evaporated under vacuum. Sugar oximes were formed using 2.5% hydroxylamine chloride in pyridine and heated to 70 ºC for 30 min. After reaction, samples were persilylated using hexamethyldisilazane (HMDS) and trifluoroacetic acid (TFA) at 50 ºC for 30 min and centrifuged at 7000 g for 5 min [24]. Two loaded chitosan impregnated under the same conditions were analyzed.

2.6.2. GC analysis

In order to determine the amount of lactulose loaded, the resulting solutions were analyzed in an Agilent Technologies 7890A gas chromatograph equipped with a flame ionisation detector (FID), using nitrogen as carrier gas. The trimethylsilyl oxime (TMSO) derivatives prepared, as described by Sanz et al., 2004 [24], were separated using an HP-5 MS fused-silica capillary column (30 m x 0.32 mm i.d. x 0.25 µm film thickness) coated with 5% phenylmethylsilicone (J&W Scientific, CA, USA). The carrier gas flow rate was 1 mL min^{-1}. Oven temperature was held at 180 ºC for 11 min, and raised to 276 ºC at a heating rate of 3 ºC min^{-1}. The injector and detector temperatures were 280 and 325 ºC, respectively. Injections were made in the split mode (1:40). Data acquisition and integration

were performed using Agilent ChemStation MSD software (Wilmington, USA). Quantitative data were calculated from FID peak areas of lactulose relative to phenyl-β-D-glucoside (internal standard). Calibration was obtained by using of standard solutions of lactulose over the expected concentration range in chitosan extracts.

2.7. Ninhydrin method

The amount of free amino groups before and after impregnation was determined by the ninhydrin method. To 0.5 ml of chitosan solution in diluted acetic acid (1 wt% in water) (in duplicate), 0.5 ml of the ninhydrin reagent was added. The ninhydrin reagent was freshly prepared on the day of the assay by adding 4M lithium acetate buffer (10 ml) to 0.8 g ninhydrin and 0.12 g hydrindantin in 30 ml DMSO [25]. The vials were immediately capped, briefly shaken by hand, and heated in a boiling water bath for 30 min to allow the reaction to proceed. The vials were then cooled in a cold water bath and the content diluted with 5 ml of 50% (v/v) ethanol/water. The solutions were then vigorously shaken on a Vortex mixer to oxidise the excess of hydrindantin [26]. The absorbance values were measured at 570 nm with a plate reader (Biotek Power Wave XS, Izasa, Madrid, Spain), zero-set against a similarly treated blank of water. The ratio of free amino groups in the sample was calculated from a standard calibration curve made with the chitosan whithout lactulose.

3. Results and discussion

3.1. Characterization of the chitosan samples

As mentioned, the possibility of preparing lactulose-loaded chitosan by supercritical fluid impregnation was evaluated in this work considering two chitosan forms: scaffolds

238 and microspheres. Figure 2 shows two digital photographs of the dry chitosan samples
239 obtained. Chitosan scaffolds prepared by freeze-drying consist of a porous structure while
240 chitosan microspheres obtained by supercritical drying resemble spherical particle with size
241 varying from 1 to 2 mm, with a high surface contact. Experiments conducted to determine
242 porosity and surface contact area allow confirming the visual observation of the images.

243 Chitosan samples were also characterized by SEM and MIP. Characteristics and
244 morphology of chitosan surface was observed by scanning electron microscopy. Later,
245 mercury intrusion porosimetry was used to provide information about porosity, pore size
246 and surface area. Figure 3 (a) and (b) shows the SEM micrographs obtained for the dry
247 chitosan samples before impregnation; scaffolds consisted on fibers or leafs regularly
248 distributed in layers showing its highly porous structure consisting of interconnected pores,,
249 while the prepared chitosan microspheres showed a typical spherical form with rough
250 surface and compact structure. Porosity analysis demonstrated that scaffolds and
251 microspheres have 98.3% and 88.9% porosity, respectively. The differential intrusion data
252 (not shown) suggest a high variability in the pore size distribution for both scaffolds and
253 microspheres, the mode pore diameter values found by MIP were 56.8 μm and 57.04 μm,
254 respectively, almost identical size for both chitosans, but with very different pore
255 morphology as seen in the SEM images. The surface contact area was measured to be 3.60
256 $m^2\ g^{-1}$ for scaffolds and 111 $m^2\ g^{-1}$ for microspheres.

257 On the other hand, FT-IR spectroscopy technique was used to determine the
258 characteristic bands of chitosan structure and to estimate the degree of deacetylation [27].
259 Spectral patterns of the chitosans obtained in this study were similar to those reported by
260 Brugnerotto et al., 2001 [28]. By considering the ratio between absorption bands at 1320

261 cm^{-1} and 1420 cm^{-1}, deacetylation was calculated to be close to 90% for low MW chitosan
262 and near 85% for medium MW chitosan.
263
264 *3.2. Chitosan impregnation yield*
265 Two different sets of experiments were performed to evaluate supercritical fluid
266 impregnation. First, preliminary experiments were carried out to study some of the
267 parameters that affect the impregnation process such as impregnation mode (batch or
268 continuous), contact time and depressurization rate. These experiments were carried out at
269 fixed conditions of 100 bar pressure and 100 ºC temperature, and using 6 wt% cosolvent
270 consisting on ethanol:water 95:5 v/v. These operating conditions corresponded to a
271 maximum lactulose solubility in the supercritical fluid, equal to 0.4058 mg g^{-1} [23]. The
272 chitosan:lactulose ratio was kept constant and equal to 10:1. Average depressurization rates
273 were between 0.60 and 3.3 bar/min, depending on the operation mode. Results expressed as
274 impregnation yield (%), obtained by GC analysis, are listed in Table 1. The impregnation
275 yield (%) is defined as the relative quantity of lactulose in an impregnated chitosan sample,
276 expressed in w/w percentage.
277 As can be seen, impregnation yields were, in general, quite acceptable considering
278 the solubility of lactulose in the impregnation mixture (SC-CO_2 + ethanol:water (95:5) at
279 6%) [23]; other authors reported smaller impregnation yields for drugs with similar
280 solubilities in the supercritical media impregnated over chitosan [3] or other polymeric
281 matrixes [29]. Reference works by Duarte and co-workers [3,29] describe the different
282 mechanisms involved in an impregnation process using supercritical fluids; these complex
283 mechanisms include interactions between the solute (lactulose), the carrier (carbon
284 dioxide), the co-solvent (ethanol:water 95:5 at 6%) and the matrix (chitosan scaffold or

chitosan microspheres). The relative strength of all binary interactions will contribute to the final partitioning of the solute between the carrier/co-solvent and the matrix.

For the system lactulose/CO2+ethanol:water/chitosan, results obtained were in agreement with those reported by other authors in which impregnation yields increased with the impregnation contact time, as expected, in batch conditions [3].

As mentioned previously, Kazarian [30] distinguished two mechanisms of impregnation assisted by supercritical fluids. The first mechanism corresponds to a simple deposition of the compound when the fluid leaves the swollen matrix; it concerns mostly solutes with a relatively high solubility in the fluid and it is specific to impregnation carried out on a matrix subjected to swelling upon exposure to a supercritical fluid. In this mechanism, the solute is solubilized in carbon dioxide and the polymer is exposed to the solution for a predetermined period followed by controlled depressurization of the system; when the system is depressurized, the carbon dioxide molecules leave the polymer matrix while the solute molecules remain trapped inside. In this case, it is expected a higher degree of impregnation when more depressurization cycles are involved. The second mechanism, not specific of supercritical fluids impregnation, involves weak chemical interactions (like van der Waals's interactions) between the solute and the matrix, that would favor the preferential partitioning of the solute within the polymer phase; this mechanism would not depend on swelling.

By analyzing the results on Table 1, it can be seen that scaffolds impregnation increased by 3.5 times when impregnation time increased from 1 to 3 h. On the other hand, when an increase in contact time was tested considering 3 cycles of 1 h/each (3 hours total), results for microspheres showed the same trend (from 0.5 % to 0.65 %) but in lower extent than when increasing the contact time continuously. Therefore, the results shown in Table 1

support the idea that the second impregnation mechanism described by Kazarian is the one controlling the impregnation process, for the particular case presented in this work, and therefore it is expected that interactions could be established between the carbonyl group of the reducing sugar and the amine groups of the polymer. Our results are in agreement with those reported by Duarte et al., 2009 [3] for the impregnation of chitosan scaffolds with dexamethasone.

On the other hand, comparing the impregnation yield (%) obtained in the experiments performed at the same operational conditions (P, T and time of contact) both in a batch and in a continuous mode, it can be observed that a continuous flow of the supercritical fluid through the impregnation cell provided higher impregnation yields compared to the batch process. These results are in contrast with those reported by Duarte et al. [3,29] although in these works authors suggested that the lower yield would be a consequence of an excessive CO_2 flow rate that did not provide an appropriate contact time. In our case, carbon dioxide flow rate was kept constant at around 1.2 g/min; this value seems to provide an adequate flow, allowing enough contact time and leaving the lactulose trapped inside the polymer matrix.

By comparing both, impregnation of scaffolds and microspheres under the same conditions (see Table 1, continuous mode), it can be seen that microspheres impregnation is faster than scaffolds impregnation; this observation is in agreement with the microsphere internal structure shown in Figure 3 c), where it can be seen that microspheres have lower porosity than scaffolds (Figure 3 a) and therefore, the interaction is faster since the solute does not enter the matrix structure.

Considering the results obtained, continuous operation mode at 1.2 g/min CO_2 flow rate, 60 minutes contact time and 3.3 bar/min depressurization rate were selected to

perform the second set of experiments to study the effect of lactulose solubility on impregnation yield on both, chitosan scaffolds and microspheres. To carry out these experiments, the ratio chitosan:lactulose was increased to 2:1, and kept constant throughout all the second set of experiments, in order to promote the availability of lactulose. Since solubility seems to be one of the main factors controlling the impregnation process by its effect on interactions such as SCF/lactulose/co-solvent and SCF/lactulose/matrix, several impregnation conditions were selected providing different experimental solubilities [23]. Selected conditions and results obtained are shown in Table 2.

First of all, the operational conditions were selected considering medium and high experimental solubilities of lactulose in SC-CO_2 + ethanol:water (95:5) (v/v) as co-solvent, according to the previous results obtained in our research group [23]; these results showed that the isothermal solubility of lactulose exhibited a minimum with pressure, thus providing the maximum solubility at higher temperatures (100 °C) at either lower and higher pressure (100 and 300 bar, respectively) and considering lower and medium amounts of co-solvent (that is, 6 and 14 %). Since the co-solvent seems to have a strong influence on the impregnation yield [20], two different systems were tested considering ethanol:water (95:5) at 6 and 14 wt %.

First observation can be drawn from the comparison between results on Table 1 and 2, obtained at the same operational conditions, from these results it is easily inferred that when the ratio chitosan:lactulose increased, impregnation yield (%) also increased for both, scaffolds and microspheres, due to the major availability of lactulose to be impregnated on a minor amount of chitosan.

As can be seen for experiments carried out at 6% and 14% of co-solvent with chitosan scaffolds, an increase of solubility (when changing conditions from 60 to 100°C)

involves an increase in the impregnation yield [20]; this increase is more important when working with 14% of co-solvent, even if, at these conditions, solubilities of lactulose are lower (in the range of 0.12-0.16 mg/g). This fact can be explained by a higher solubility of CO_2 in the polymer with the increase of co-solvent percentage and therefore, better possibilities of interaction between the solute and the matrix (lactulose-chitosan), leading to higher yields. These results demonstrated the usefulness of these studies to experimentally determine the best conditions to carry out impregnation of chitosan with a valuable solute such as lactulose because the highest solubility does not always involve the highest impregnation yield.

As for microspheres, even if the behavior is quite similar in terms of solubility of lactulose vs impregnation yield (%) when 6% of co-solvent is used, the study of the global results seemed to point out that, once a maximum value is reached (around 4%), no further impregnation can be obtained even modifying the operation conditions. This could be due to the different structure of the microspheres that would provide a lower exposition of the functional groups of the chitosan to the solute, thus precluding a higher impregnation extent in this type of matrix.

SEM images did not allow us to conclude if lactulose was deposited on the surface or into the core of the matrix during impregnation, since images were almost identical before and after the impregnation process (images after impregnation not shown) and therefore, no clear conclusions could be drawn. On the other hand, considering the low concentration of lactulose that has been impregnated, it is expected not to have conclusive information by using SEM or even FT-IR analysis. In this sense, FT-IR was also used, without conclusive results, to establish possible interactions between the carbonyl group of lactulose and the amine groups of chitosan [31]. As mentioned, spectra were almost

identical before and after the impregnation process, probably due to the low concentration of lactulose in the samples.

To study the interactions chitosan-lactulose in the impregnated samples, the ninhydrin method was used; by using this method, the amount of free amino groups before and after impregnation was determined. Results showed that only in the experiments with chitosan scaffolds at 100ºC (impregnated in a continuous mode using 2:1 chitosan:lactulose ratio at 100 bar pressure and 6 or 14% of co-solvent) a decrease of approximately 40% of the free amino groups was observed. This samples were those with the highest impregnation yields and also showed extensive browning, demonstrating the extent of the Maillard reaction. No interaction was observed for chitosan microspheres, probably due to the low concentration of lactulose impregnated in the matrix, a lower exposition of the amino groups of the microspheres of chitosan and their closed (or more compact) structure. The occurrence of the Maillard reaction on this type of impregnation processes can provide with new chitosan-lactulose derivatives. Other authors have reported that the Maillard reaction can be successfully employed to develop products from chitosan, exhibiting improved properties [12,17].

4. Conclusions

In this work we demonstrated the usefulness of the supercritical impregnation process to successfully impregnate chitosan with lactulose, a prebiotic sugar able to provide chitosan with improved properties. Various chitosan forms were tested, such as scaffolds obtained by freeze-drying and microspheres dried under SC-CO_2 conditions. As demonstrated in the present work, the mechanism controlling the impregnation process for the chitosan and the disaccharide (lactulose) studied in this work, is the chemical interaction (Van der Waals

interactions) between lactulose and the amino groups of chitosan. Different experimental conditions were tested and the results suggested that the best impregnation conditions for chitosan scaffolds were obtained working at 100 bar, 100 ºC and 14 wt% cosolvent (ethanol:water 95:5), under continuous operation mode at considering a contact time equal to 60 minutes, a depressurization rate of 3.3 bar/min and a ratio chitosan:lactulose equal to 2:1. As for chitosan microspheres, similar optimum conditions were observed but, in this case, the lactulose impregnation yield reached a maximum at 6 wt% cosolvent (ethanol:water 95:5). The occurrence of the Maillard reaction was also measured for chitosan scaffolds with the highest impregnation yield, suggesting that it is possible not only to control the degree of impregnation but also the extension of the reaction, depending on the operation conditions. Thus, results demonstrated that supercritical CO_2 impregnation can be consider as a new environmentally friendly technique effective for the impregnation of chitosan with mono- or disaccharides.

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Figure captions

Figure 1. Schematic diagram of the supercritical fluid impregnation apparatus used in this work. The equipment consists in a heated high pressure cell in which scCO2 liquefied + co-solvent is introduced, followed by a depressurization system.

Figure 2. Digital pictures of lyophilized chitosan scaffolds (a) and chitosan microspheres after $scCO_2$ drying (b).

Figure 3. SEM images of a) external surface of a lyophilized chitosan scaffold (800x) (b) external surface of a $scCO_2$ dried chitosan microsphere (50x) (c) internal structure of a scCO2 dried chitosan microsphere (800x).

Table 1

Results of the preliminary impregnation experiments performed in scaffolds and microspheres chitosan form.

Chitosan form	*CO_2 flow (g min^{-1})*	*Impregnation time (min)*	*Depressurization rate (bar / min)*	**Impregnation yield (%)**
Scaffolds	Batch	60	1	0.40
	Batch	180	0.6	1.45
	1.2	60	3.3	0.65
Microspheres	Batch	60	1	0.50
	Batch	60 (*3 cycles)	1	0.65
	1.2	60	3.3	1.90

Table 2

Operational conditions and results of the impregnation experiments performed on chitosan scaffolds and microspheres. Fixed conditions: impregnation pressure, 100 bar, continuous operation mode at 1.2 g/min CO_2 flow rate, 60 minutes contact time and 3.3 bar/min depressurization rate.

T (ºC)	*wt% cosolvent*	*Lactulose solubility (mg g^{-1})*	**Impregnation yield (%)**	
			Scaffolds	Microspheres
60	6	0.2508	0.24	2.94
100	6	0.4058	3.58	3.96
60	14	0.1224	2.54	3.92
100	14	0.1622	8.61	2.45