

1 **Supercritical CO₂ impregnation of lactulose on chitosan: A comparison between**
2 **scaffolds and microspheres form.**

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10

11 **Abstract**

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

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

26

27 *Keywords:* Chitosan, Scaffolds, Microspheres, Supercritical CO₂, Impregnation, Lactulose

28

29 **1. Introduction**

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

35 The degree of deacetylation and molecular weight of chitosan determines
36 physicochemical properties and biological activities of chitosan [2]. Chitosan has been
37 processed in several forms, namely, scaffolds and microspheres, by a variety of methods.
38 Chitosan scaffolds can be prepared by freeze-drying of a chitosan gel solution [3] while
39 microspheres can be obtained by drying gel beads of the natural polymer under
40 supercritical CO₂ conditions; this particular method makes the accessibility of chitosan
41 functional groups easy [4,5].

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

48 consolidated mainly due to the limited solubility of chitosan in neutral and basic solutions
49 [10,11]. However, it is known that incorporation of 3–30% of mono- or disaccharide
50 residues into the chitosan molecule changed the solubility of its derivatives at pH higher
51 than the apparent acidity constant of chitosan amino groups (6.3–6.7) [12].

52 Lactulose (4- α -D-galactopyranosyl-D-fructose) is a synthetic ketose disaccharide
53 obtained from lactose by alkaline isomerization [13]. Lactulose is a prebiotic carbohydrate
54 with ability to stimulate the growth and activity of bifidobacteria and lactobacilli present in
55 the gastrointestinal tract, performing many important functions such as protection from
56 food-borne illnesses and allergies, regulating hormone balance, and enhancing immunity
57 [14,15].

58 Supercritical fluids (SCFs) are considered an attractive alternative to organic
59 solvents for polymer processing [16]. Besides its environmental friendly status, the main
60 reason for using SCFs in polymer processing comes from the opportunity to utilize SCFs
61 favorable properties such as high diffusivities, low viscosities, and near zero surface tension
62 which allow a rapid penetration into a high variety of matrices. Although there is a wide
63 range of compounds that can be used as supercritical fluids, carbon dioxide (SC-CO₂) is, by
64 far, the most used due to its moderate critical temperature (31 °C) and pressure (72 bar), its
65 cheapness and its GRAS (generally recognized as safe) status by FDA (Food and Drug
66 Administration) and EFSA (European Food Safety Authority). Another advantage is that
67 CO₂ is gaseous at room temperature and pressure which provides solvent-free polymeric
68 matrices.

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

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

82 Impregnation efficiency results from a complex mechanism that involves
83 interactions between the solute (lactulose), the mobile phase (carbon dioxide + cosolvent)
84 and the matrix (chitosan). The relative strength of all binary interactions will contribute to
85 the final partitioning of the solute between the mobile phase and the matrix [3]. The phase
86 behavior of different polymers, such as chitosan, in supercritical carbon dioxide has been
87 widely studied in recent years [5,21]. It is also known from literature that solute solubility
88 in CO₂ will increase when using a cosolvent with the same polar characteristics of the
89 solute [22]. Undoubtedly, knowing the solubility of the solute in the supercritical media is
90 crucial to optimize impregnation conditions. In this sense, solubility of lactulose in SC-CO₂
91 with (ethanol + water) as cosolvent at certain operational conditions (pressure and
92 temperature) has been previously reported by Montañes et al., 2009 [23].

93 Thus, the main goal of this work was to study and optimize the impregnation of
94 lactulose into two chitosan forms: chitosan scaffolds and chitosan microspheres.
95 Supercritical fluid impregnation methodology has been used employing CO₂ and

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

98

99 **2. Materials and methods**

100 *2.1. Materials*

101 Two types of chitosan were purchased from Sigma-Aldrich (Madrid, Spain): a low
102 molecular weight (150 kDa) and a medium molecular weight (350 kDa). Lactulose (98%
103 purity), internal standard (phenyl- β -D-glucoside), methanol and derivatizing reagents
104 (hydroxylamine hydrochloride, hexamethyldisilazane and trifluoroacetic acid) were also
105 obtained from Sigma-Aldrich. Acetic acid and sodium hydroxide were purchased from
106 Panreac (Barcelona, Spain), ethanol absolute was from Prolabo (Madrid, Spain), and
107 pyridine was supplied by Merck (Darmstadt, Germany). Ultrapure water quality (18.2
108 M Ω cm) with 1–5 ppb total organic carbon (TOC) and <0.001 EU/mL pyrogen levels was
109 produced in-house using a laboratory water purification Milli-Q Synthesis A10 system
110 from Millipore (Billerica, USA). Carbon dioxide (CO₂) liquefied at high pressure used in
111 supercritical fluid impregnation was supplied by Praxair (Madrid, Spain). Washed glass
112 wool chemically pure was acquired from Panreac.

113

114 *2.2. Preparation of lyophilized chitosan scaffolds*

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

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

121

122 *2.3. Preparation of scCO₂ dried chitosan microspheres*

123 A solution of 1 wt% chitosan (medium molecular weight) in a diluted acetic acid
124 solution (2 wt% in water) was prepared. Total dissolution was obtained by stirring during 5
125 h at room temperature. This solution was added dropwise into a sodium hydroxide solution
126 (5 wt% in water) through a burette. The chitosan microspheres were repeatedly washed
127 with ultrapure water until neutral pH, and then dehydrated by immersion in a series of
128 successive ethanol–water baths of increasing alcohol concentration (10, 30, 50, 70, 90, and
129 100%) for 15 min each. Finally, the microspheres were dried under supercritical CO₂
130 conditions (74 bars, 32 °C) during 2 h in the Suprex Prep Master apparatus described below
131 [4].

132

133 *2.4. Supercritical solvent impregnation (SSI) process*

134 The supercritical impregnation apparatus used to perform the experiments is
135 schematically presented in Figure 1. The equipment is based on a Suprex Prep Master
136 (Suprex Corporation, Pittsburg, PA, USA) with several modifications. It has a thermostatic
137 oven heated by air convection where the impregnation cell (with approximately 10 cm³ of
138 internal volume) containing the sample is placed. A pre-heater system was employed by
139 placing a heating coil inside a glycerin bath (JP Selecta Agimatic N, JP Selecta S.A.,
140 Abrera, Spain) to guarantee that the fluid employed in all the experiments reaches the high
141 pressure vessel at the target temperature. The system is also equipped with a Suprex solvent
142 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 75 μ 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 CO₂ 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

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

171 The operating pressure and temperature and the amount of cosolvent for each
172 experiment were established considering the solubility of lactulose (saturated environment)
173 in the mixture of compressed fluid and cosolvent, and according to data reported previously
174 [23].

175

176 *2.5. Chitosan characterization procedures*

177 *2.5.1. Scanning electron microscopy (SEM)*

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

182 *2.5.2. Mercury Intrusion Porosimetry (MIP)*

183 Porosity measurements (pore size, surface area, % porosity) were carried out in
184 chitosan samples using a mercury intrusion porosimeter PoreMaster Series 60 model
185 (Quantachrome Instruments, Boynton Beach, Florida, USA). The MIP was performed and
186 analyzed under standard conditions (Hg surface tension $\sigma = 480.00 \text{ erg/cm}^2$, Hg contact
187 angle $\Theta = 140.00^\circ$, pressure range 0-50 PSIA for low pressure and 20-60000 PSIA for high
188 pressure experiments).

189 *2.5.3. Fourier transform infrared spectroscopy (FT-IR)*

190 Infrared spectra were obtained with an FT-IR spectrometer (Perkin Elmer Spectrum
191 One, California, USA) by using the KBr pellet method and were recorded by an average of
192 64 scans at a resolution of 4 cm⁻¹.

193

194 *2.6. Quantitative gas chromatography (GC) analysis of lactulose loading*

195

2.6.1. Sample preparation

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

204

2.6.2. GC analysis

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

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

218

219 *2.7. Ninhydrin method*

220 The amount of free amino groups before and after impregnation was determined by
221 the ninhydrin method. To 0.5 ml of chitosan solution in diluted acetic acid (1 wt% in water)
222 (in duplicate), 0.5 ml of the ninhydrin reagent was added. The ninhydrin reagent was
223 freshly prepared on the day of the assay by adding 4M lithium acetate buffer (10 ml) to 0.8
224 g ninhydrin and 0.12 g hydrindantin in 30 ml DMSO [25]. The vials were immediately
225 capped, briefly shaken by hand, and heated in a boiling water bath for 30 min to allow the
226 reaction to proceed. The vials were then cooled in a cold water bath and the content diluted
227 with 5 ml of 50% (v/v) ethanol/water. The solutions were then vigorously shaken on a
228 Vortex mixer to oxidise the excess of hydrindantin [26]. The absorbance values were
229 measured at 570 nm with a plate reader (Biotek Power Wave XS, Izasa, Madrid, Spain),
230 zero-set against a similarly treated blank of water. The ratio of free amino groups in the
231 sample was calculated from a standard calibration curve made with the chitosan without
232 lactulose.

233

234 **3. Results and discussion**

235 *3.1. Characterization of the chitosan samples*

236 As mentioned, the possibility of preparing lactulose-loaded chitosan by supercritical
237 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 $\text{m}^2 \text{g}^{-1}$ for scaffolds and 111 $\text{m}^2 \text{g}^{-1}$ 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 $100 \text{ }^\circ\text{C}$ 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

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

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

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

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

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

315 On the other hand, comparing the impregnation yield (%) obtained in the
316 experiments performed at the same operational conditions (P, T and time of contact) both in
317 a batch and in a continuous mode, it can be observed that a continuous flow of the
318 supercritical fluid through the impregnation cell provided higher impregnation yields
319 compared to the batch process. These results are in contrast with those reported by Duarte
320 et al. [3,29] although in these works authors suggested that the lower yield would be a
321 consequence of an excessive CO₂ flow rate that did not provide an appropriate contact time.
322 In our case, carbon dioxide flow rate was kept constant at around 1.2 g/min; this value
323 seems to provide an adequate flow, allowing enough contact time and leaving the lactulose
324 trapped inside the polymer matrix.

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

331 Considering the results obtained, continuous operation mode at 1.2 g/min CO₂ flow
332 rate, 60 minutes contact time and 3.3 bar/min depressurization rate were selected to

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

341 First of all, the operational conditions were selected considering medium and high
342 experimental solubilities of lactulose in SC-CO₂ + ethanol:water (95:5) (v/v) as co-solvent,
343 according to the previous results obtained in our research group [23]; these results showed
344 that the isothermal solubility of lactulose exhibited a minimum with pressure, thus
345 providing the maximum solubility at higher temperatures (100 °C) at either lower and
346 higher pressure (100 and 300 bar, respectively) and considering lower and medium
347 amounts of co-solvent (that is, 6 and 14 %). Since the co-solvent seems to have a strong
348 influence on the impregnation yield [20], two different systems were tested considering
349 ethanol:water (95:5) at 6 and 14 wt %.

350 First observation can be drawn from the comparison between results on Table 1 and
351 2, obtained at the same operational conditions, from these results it is easily inferred that
352 when the ratio chitosan:lactulose increased, impregnation yield (%) also increased for both,
353 scaffolds and microspheres, due to the major availability of lactulose to be impregnated on
354 a minor amount of chitosan.

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

357 involves an increase in the impregnation yield [20]; this increase is more important when
358 working with 14% of co-solvent, even if, at these conditions, solubilities of lactulose are
359 lower (in the range of 0.12-0.16 mg/g). This fact can be explained by a higher solubility of
360 CO₂ in the polymer with the increase of co-solvent percentage and therefore, better
361 possibilities of interaction between the solute and the matrix (lactulose-chitosan), leading to
362 higher yields. These results demonstrated the usefulness of these studies to experimentally
363 determine the best conditions to carry out impregnation of chitosan with a valuable solute
364 such as lactulose because the highest solubility does not always involve the highest
365 impregnation yield.

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

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

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

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

397

398 **4. Conclusions**

399 In this work we demonstrated the usefulness of the supercritical impregnation process to
400 successfully impregnate chitosan with lactulose, a prebiotic sugar able to provide chitosan
401 with improved properties. Various chitosan forms were tested, such as scaffolds obtained
402 by freeze-drying and microspheres dried under SC-CO₂ conditions. As demonstrated in the
403 present work, the mechanism controlling the impregnation process for the chitosan and the
404 disaccharide (lactulose) studied in this work, is the chemical interaction (Van der Waals

405 interactions) between lactulose and the amino groups of chitosan. Different experimental
406 conditions were tested and the results suggested that the best impregnation conditions for
407 chitosan scaffolds were obtained working at 100 bar, 100 °C and 14 wt% cosolvent
408 (ethanol:water 95:5), under continuous operation mode at considering a contact time equal
409 to 60 minutes, a depressurization rate of 3.3 bar/min and a ratio chitosan:lactulose equal to
410 2:1. As for chitosan microspheres, similar optimum conditions were observed but, in this
411 case, the lactulose impregnation yield reached a maximum at 6 wt% cosolvent
412 (ethanol:water 95:5). The occurrence of the Maillard reaction was also measured for
413 chitosan scaffolds with the highest impregnation yield, suggesting that it is possible not
414 only to control the degree of impregnation but also the extension of the reaction, depending
415 on the operation conditions. Thus, results demonstrated that supercritical CO₂ impregnation
416 can be consider as a new environmentally friendly technique effective for the impregnation
417 of chitosan with mono- or disaccharides.

418

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512 **Figure captions**

513

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

517

518 Figure 2. Digital pictures of lyophilized chitosan scaffolds (a) and chitosan microspheres
519 after scCO₂ drying (b).

520

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

524

525 **Table 1**

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

528

<i>Chitosan form</i>	<i>CO₂ flow (g min⁻¹)</i>	<i>Impregnation time (min)</i>	<i>Depressurization rate (bar / min)</i>	Impregnation yield (%)
	Batch	60	1	0.40
Scaffolds	Batch	180	0.6	1.45
	1.2	60	3.3	0.65
	Batch	60	1	0.50
Microspheres	Batch	60 (*3 cycles)	1	0.65
	1.2	60	3.3	1.90

529

530

531 **Table 2**

532 Operational conditions and results of the impregnation experiments performed on chitosan
533 scaffolds and microspheres. Fixed conditions: impregnation pressure, 100 bar, continuous
534 operation mode at 1.2 g/min CO₂ flow rate, 60 minutes contact time and 3.3 bar/min
535 depressurization rate.

536

<i>T</i> (°C)	<i>wt%</i>	<i>Lactulose solubility</i>	Impregnation yield (%)	
	<i>cosolvent</i>	(<i>mg g⁻¹</i>)	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

