On the solubility of chitosan in aqueous acetic acid and pressurized (water + carbon dioxide)

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ABSTRACT

Medium-molar-mass chitosan was purified and characterized by size exclusion chromatography, viscosity, 1H-NMR and UV spectrometry. As a result, a molar mass distribution of M_z (1,130.4 ± 57.1 kDa) > M_w (339.7 ± 11.2 kDa) > M_v (302.92 ± 26.68 kDa) > M_n (58.2 ± 8.1 kDa), and a degree of acetylation of *ca.* 0.150 and 0.197 ± 0.003 using 1H-NMR and UV spectrometry, respectively, were obtained. An experimental apparatus for measuring high-pressure equilibrium data up to 30 MPa, was assembled and validated regarding the degree of protonation of chitosan in aqueous acetic acid and the pH of pressurized (water + carbon dioxide) at 35, 42, and 50 ºC. Finally, based on the validation experiments, new chitosan solubility data in pressurized (water + carbon dioxide) were obtained at 25 ºC and pressures from *ca*. 95 to *ca.* 1000 psi.

Keywords: solubility, chitosan, high pressure, water, carbon dioxide, spectroscopy

INTRODUCTION

Chitosan, the N-deacetylated derivative of Chitin, which is the second most abundant and important polymer in nature after cellulose, is mainly found in the exoskeleton of crustaceans or in the cell walls of fungi and yeasts [1]. Both are aminopolysacharides constituted by N-acetyl glucosamine and glucosamine units connected by β (1-4) linkage and distributed randomly throughout the polymer chain. When the glucosamine unit concentration (also known as degree of deacetylation) exceeds 50%, the copolymer is recognized as Chitosan, otherwise as Chitin. These substances differ in many of their properties, especially in solubility.

Chitosan is a non-toxic, biocompatible, biodegradable, and relatively easy-to-manufacture copolymer, which has many valuable properties due to its physical configuration and chemical structure. Its anti-inflamatory, antibacterial, mucoadhesive and wound healing properties make it very attractive for biomedical applications [2–5]. Hence, despite the vast information available about chitosan, it is still today one of the most studied compounds in science and engineering applied to medicine.

One of the key properties for designing and developing products in many applications is solubility. The solubility of chitosan depends on many factors such as molar mass

distribution, degree of acetylation, pH and temperature, among others. Several efforts have been done regarding solubility measurements of chitosan in acidic media (e.g. using aqueous acetic acid and hydrochloric acid). According to Rinaudo et al. [6,7], Chitosan can be solubilized when a degree of protonation higher than 50% is reached; nevertheless, there is not enough information in the literature about the solubility of chitosan in other acidic media, such as water pressurized with carbon dioxide.

In this work we present solubility measurements of medium molar mass chitosan in aqueous acetic acid and water saturated with compressed carbon dioxide. To carry out such equilibrium measurements, we assembled an experimental apparatus and validated its performance by determining solubility data of chitosan in aqueous acetic acid at atmospheric pressure, and pH data of water in the presence of compressed carbon dioxide at 35, 42 and 50 ºC. Results from those experiments were used to experimentally determine the chitosan solubility in water, in the presence of compressed carbon dioxide at different pressures.

MATERIALS AND METHODS

Materials Medium-molar-mass chitosan was obtained from Sigma-Aldrich (Saint louis, MO, USA). Glucosamine hydrochloride (99,9 ± 0,4 wt.%) and N-acetylglucosamine (98,6 ± 0,2 wt.%) were obtained from Sigma-Aldrich (Laramie, MY,USA). Pullulan standards with peak molar mass from 1.3 to 21.0 kDa were obtained from Sigma-Aldrich (Buchs, Switzerland) and those with 202.9 and 501.6 kDa were obtained from American polymer standards corporation ($M_w/M_n \leq 1.6$) (Mentor, OH, USA).

Carbon dioxide (99,9 v.%) and nitrogen (99.9990 v.%) were obtained from Cryogas INDURA (Sibaté, Cund., Colombia). Water was purified in our laboratory by two successive steps of filtration, followed by deionization and distillation. Glacial acetic acid, hydrochloric acid (37 wt.%), sodium chloride (>99.5 wt.%), sodium nitrate (>99.5 wt.%) and bromophenol blue were obtained from Merck (Darmstadt, Germany). Buffer solutions with pH of 1,68, 4,01, 7,01 and 10,00 (± 0,01 at 25 °C) were obtained from Hanna Instruments (Woonsocket, RI, USA). All compounds were used without further purification except for chitosan which was purified as described below.

Characterization of Chitosan

Purification. Impurities in commercial chitosan such as proteins, ashes, salts, among others [8], were eliminated according to the following procedure. *ca.* 5 g of chitosan were dissolved in *ca.* 500 mL of 2 wt.% aqueous acetic acid, stirring during 24 h. Resulting chitosan solution was centrifuged at 4000 rpm, and the supernatant was passed through 8.0 and 0.8 μm cellulose nitrate filters, respectively, using a vacuum filtration system. In order to precipitate chitosan, 10 wt.% sodium hydroxide solution was added dropwise to the purified chitosan solution until neutralization (pH ~ 8). 5 or 3 successive washes using distilled water or ethanol/ water mixtures (96:4 v.%), respectively, were used to eliminate the remaining salt. Finally, 14 h of oven-drying at 55 ºC were necessary to obtain the purified chitosan as a yellowish solid, which in turn was subjected to a cryogenic milling using liquid nitrogen in order to obtain different particle sizes. Due to its hygroscopic nature, chitosan was oven-dried before each test.

Molar mass distribution. An approximation to the molar mass distribution of chitosan was

made using size exclusion chromatography (SEC) [9,10], and capillary viscosimetry [8,11]. The number average (M_n), the weight average (M_w) and the Z average (M_z) molar masses were determined using SEC, as follows. A calibration curve was prepared using 6 pullulan standards with different peak molar mass, namely 501.6, 202.9, 21.0, 9.6, 6.1 and 1.3 kDa. For both, pullulan standards and chitosan, 1.20 ± 0.01 mg of sample were dissolved in 1 mL of a 0.5 M acetic acid/ 0.15 M sodium nitrate filtered solution, after 48 h of indirect sonication. Resultant solutions were filtered using 0.43 μm nitrocellulose filters and 20 μL thereof were injected in an Agilent Infinity series chromatograph, equipped with an isocratic pump, two packed columns: Shodex OHPak SB-805 and -806 (8.0x300nm, 13 μm), and a refractive index detector. Mobile phase flow and temperature were constant during the analyses and equals to 0.5 mL/min and 35ºC, respectively. All the analyses were run by duplicate.

Figure 1 shows a calibration curve for the determination of the molar mass distribution by size exclusion chromatography. The curve represents the behavior of the decimal logarithm of the peak molar mass (log M_p) as a function of retention time (RT) in the chromatographic columns. Figure 2 shows the molar mass distribution of commercial medium molar mass chitosan. As expected, the distribution dispersity is high, since the size of the commercial chitosan chains is diverse. From these results number average (M_n), weight average (M_w) and Z average (M_z) molar mass were obtained, as well as the polydispersity index. These values are shown in Table 1.

On the other hand, the viscosity average molar mass (M_υ) was determined using a No. 150 Canon Fenske viscometer in a thermostatic bath at 25ºC. Chitosan solutions between 0.001 and 0.02 g/dL were prepared using a mixture of 0.1M acetic acid and 0.02M NaCl, at constant stirring during 24 h. Kinematic and intrinsic viscosity measurements were computed using the Hagen- Poiseuille equation with a kinetic energy correction [12] and the Huggins equation [13]. Finally, the viscosity average molar mass was calculated using the Mark-Houwink-Sakurada (MHS) relationship, with k and a constants equal to 3.04×10^{-7} dL/g and 1.26, respectively[11]. All measurements were performed by triplicate. As observed in Figure 3, a high correlation was obtained between experimental data and the Huggins equation. The intrinsic viscosity and the viscosity average molar mass were determined as $[\eta] = 2.451 \pm 0.272$ dL/g, and $M_\upsilon = 302.92 \pm 26.68$ kDa. As expected, results in Table 1 show that the molar mass distribution follows the sequence $M_z > M_w > M_v > M_n$.

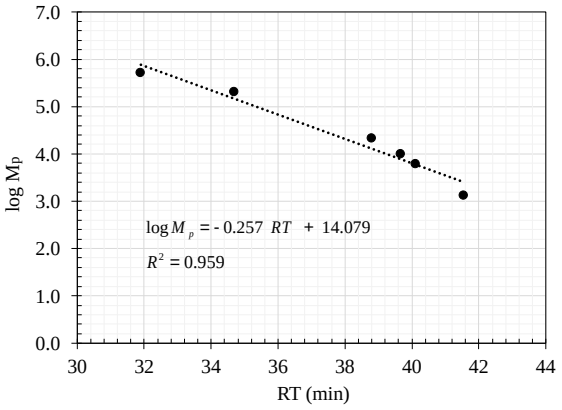

Fig. 1 Calibration curve for the determination of the molar mass distribution by size exclusion chromatography

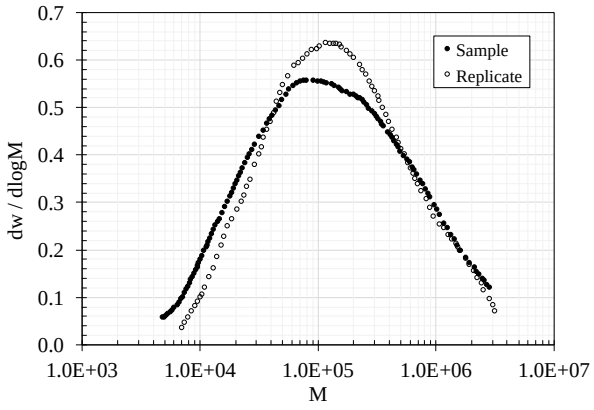

Fig. 2 Molar mass distribution of commercial medium-molar-mass chitosan

Table 1. Average molar masses and polydispersity index determined for commercial medium-molar-mass chitosan

Average molar mass	Mean value (kDa)
Mn	58.2 ± 8.1
Mw	39.7 ± 11.2
Mz	1,130.4 ± 57.1
Polidispersity index	5.9 ± 0.6

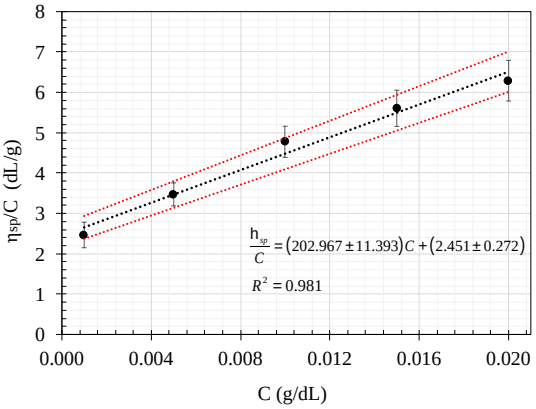

Fig. 3 Quotient between the specific viscosity of the chitosan solutions and the chitosan solutions concentration as a function of the chitosan solution concentration, using 0.1M acetic acid/ 0.02M sodium chloride as solvent, at 25 ºC.

Degree of acetylation (DA) The DA was determined by 1H-NMR and UV spectroscopy, based on [14,15], respectively. 1H-NMR analyses were performed in a Bruker Avance II UltraShield 400MHz NMR spectrometer, using 10 ± 0.1 mg of sample in 1 mL of deuterated water and two drops of trichloroacetic acid as solvent at 25ºC. All the samples were freeze-dried overnight and dissolved during 24 hours at constant stirring. Finally, the DA was calculated according to the integration method proposed by Hirai et.al in [14]:

$$DA = \frac{(1/3)\, I_{CH_3}}{(1/6)\, I_{H2\text{-}H6}} \quad (1)$$

where I_{CH_3} and $I_{H2\text{-}H6}$ correspond to the integrals of the N-acetyl groups and H2-H6 protons at 1.95 and 2.7 to 4.4 ppm, respectively.
Figure 4 shows the 1H NMR spectrum of chitosan in aqueous trifluoroacetic acid at 25ºC. The assignments were made according to [16,17]. Using equation 1, a DA= 0.150 was computed.
UV spectroscopy analyses were performed in an Evolution 60 ThermoScientific UV/vis spectrometer. A calibration curve was prepared using 8 solutions of N-acetylglucosamine and glucosamine hydrochloride standards in 0.10 M hydrogen chloride, to obtain DA from 0.05 to 0.60. All measurements were performed by triplicate at 207 nm, where the maximum absorbance was found. The DA was calculated using an equation similar to that used by Liu et .al [15], except that we measured weight instead of volume for increasing accuracy:

$$DA = \frac{m_q\, Z - 161.1\, A\, m_{s/n}}{42.1\, A\, m_{s/n} - m_q\, k} \quad (2)$$

where m_q is the mass of chitosan sample in mg, Z is the intercept of the calibration curve in Abs kg/ mmol, A is the absorbance in Abs, $m_{s/n}$ is the mass of the solution in kg, and k is the slope of the calibration curve in Abs kg/ mmol.

Figure 5 shows the calibration curve for DA measurements using UV spectroscopy, and two UV spectrums as examples, one for a mixture with DA= 0.20 and other for the purified chitosan. As observed, the linear model fitted very good the experimental data, and allowed us to estimate a degree of acetylation of 0.197 ± 0.003. This result differs 0.7 ± 0.3 % from that reported by Sigma-Aldrich, and 4.7% from that obtained by 1H NMR.

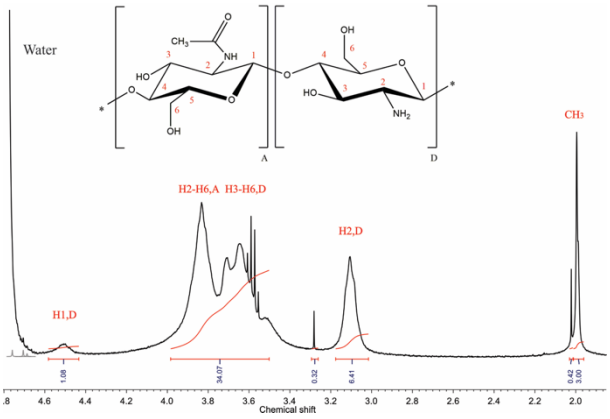

Fig. 4 1H NMR spectrum of chitosan in aqueous trifluoroacetic acid at 25ºC.

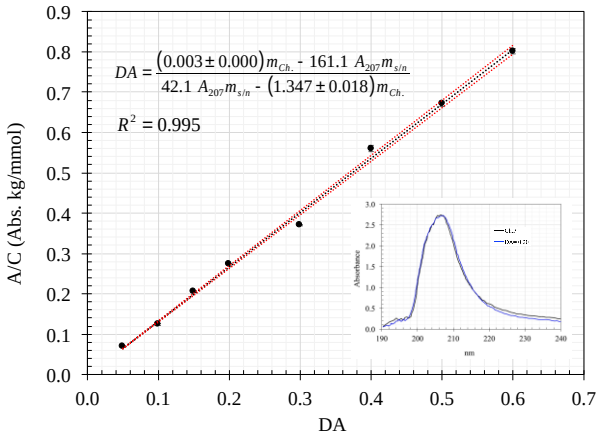

Fig. 5 Calibration curve for DA measurements using UV spectroscopy. Red dashed lines represent the error bars associated to the measurement's uncertainty.

Experimental apparatus for determination of chitosan solubility

Figure 6 shows a schematic of the experimental apparatus for the determination of chitosan solubility in acid media at low and high pressure. The apparatus is conformed by (1) a pumping zone, which feeds CO_2 into (2) a reaction system, and (3) an UV-vis spectroscopy assembly that detects changes in the absorbance of a reaction mixture until it reaches the equilibrium.

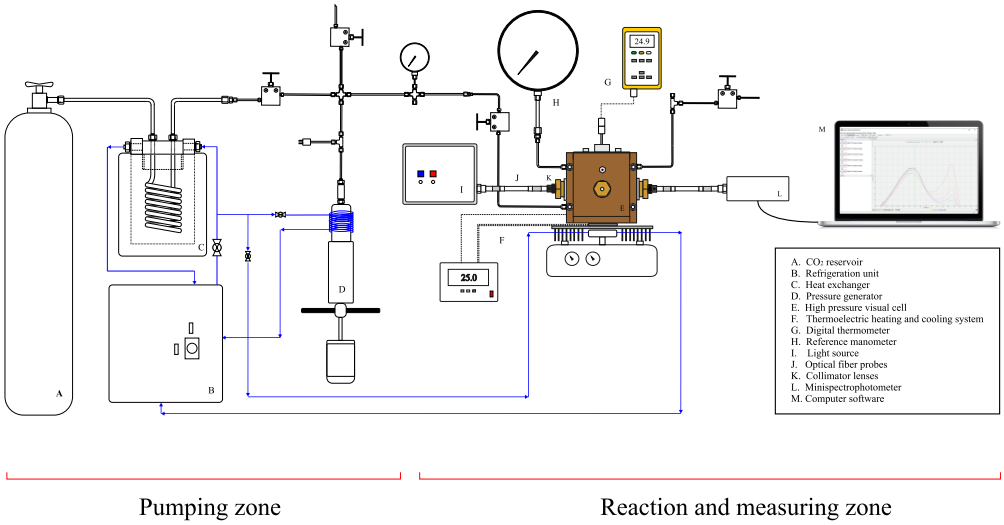

Fig. 6 Schematic of the experimental apparatus for the determination of chitosan solubility in acidic media

The reaction system consists of a high pressure visual cell (E), and a thermoelectric heating and cooling system (F). This cell is an assembly of (1) a central body made of stainless steel 316 with 4 lateral cavities, each one holding a UVFS windows, which in turn are adjusted using threaded bushings, and (2) a cap designed for holding a standard cuvette, where the reaction and equilibrium take place. Both the central body and the cap were designed and built in our lab to operate up to 30 MPa.

The reaction and equilibrium temperatures in the cuvette were measured to a precision of ± 0.1 ºC by using a K-type thermocouple and a digital thermometer, both calibrated against a high-precision RTD (FLUKE, 1512A model, ±0.0006ºC), whose calibration is in turn traceable to a NIST standard. Pressure in the visual cell was measured to a precision of ± 5 psi by using a 12 inches precision Bourdon pressure gage, calibrated against a digital manometer (GE DRUCK, DPI 104 IS model, 0 to 68950 kPa, 1 kPa subdivision, 0.05% accuracy).

Validation of the experimental apparatus

Degree of protonation of chitosan. The apparatus was first calibrated for measuring and monitoring pH in the reactive system, using solutions of chitosan in aqueous acetic acid at different concentrations, and 40.0 ± 0.1 mg of a pH-indicator solution of 0.25 wt.% bromophenol blue (BPB) in distilled water. The pH was measured in a double junction pH-meter with temperature sensor (Hanna Instruments, -2.00 to 16.00 pH, ±0.01 pH accuracy, -5.0 to 100 ºC, RI, USA), previously calibrated using buffer solutions of 1.68, 4.01, 7.01 and 10.00 pH units. Three sets of experiments were performed in order to determine the solubility of chitosan in acetic acid solutions with different pH, at 25, 35 and 50 ºC. In a typical experiment, 2.000 ± 0.001 g of an acetic acid solution (from 0.004 to 1.00 M), 40.0 ± 0.1 mg of 0.25 wt.% BPB solution, and a fixed amount of chitosan (1, 2 or 5 ($x10^{-2}$) monoM) were added to a carefully-cleaned standard cuvette. The cuvette was then held by the cap-holder, and both introduced in the visual cell. The reactive system was kept at the desired temperature and constant stirring until it reached equilibrium. The Absorbance ratio A_{440}/A_{590} was monitored during each experiment, and pH was calculated taking into account the calibration curves and verified using the pH-meter. The degree of protonation was computed following Rinaudo's approach [6].

pH measurements of pressurized water with carbon dioxide. In order to determine the pH in pressurized (water + carbon dioxide) systems, the experimental apparatus was again calibrated, but in this case using aqueous acetic acid solutions with pH ranging from 3.01 to 4.50, at 20, 25, 30 and 50 ºC, and inert atmosphere. To validate our experimental apparatus at high pressure, experiments at 35, 42 and 50 ºC, and pressures from 3.8 to 62.3 bar, were performed and the results of pH were compared with experimental data already reported in the literature [18,19]. Similar to the aforementioned on the chitosan protonation experiments, in a typical experiment, 2.000 ± 0.001 g of distilled water and 50.0 ± 0.1 mg of 0.25 wt.% BPB solution were added to a standard cuvette. The cuvette was then held by the cap-holder, and both introduced in the visual cell. The holder-cap was screwed to the central body of the visual cell, and the air inside was purged with carbon dioxide from the pumping zone, at 50 psig. After 3 minutes, pressure was increased to a desired value, while temperature was reaching a determined set-point value. The reactive system was kept at constant stirring and

the A_{450}/A_{590} absorbance ratio was monitored during each experiment. When the behavior became asymptotic, stirring was stopped and once the absorbance ratio was approximately constant (± 0.001), the pH was calculated using the calibration curves.

Chitosan solubility measurements

Based on the validation results, solubility experiments of chitosan in pressurized (water + carbon dioxide) were performed at 25 ºC. In a typical experiment for the determination of chitosan solubility, the preparation of a single run was similar to that described for the pH measurements experiments, except for the addition of a determined amount of oven-dried chitosan (10, 21, 30 and 40 ± 0.1 mg) to the water-indicator solution in the cuvette. The system was kept at constant stirring during 18 h and resting 6 h.
Solubility was determined by increasing pressure from 0 psig up to the dissolution pressure, at an arbitrary pressure pace and at a determined temperature. Once Chitosan dissolved, the experiment was stopped and repeated, charging the same amounts of the compounds, operating from the previous pressure, and with a smaller pressure pace, in order to approach the dissolution pressure with the lowest possible uncertainty. The dissolution pressure was determined to a precision of ± 5 psig.

RESULTS

Degree of protonation of chitosan. After *ca.* 24 h, the equilibrium pH and the degree of protonation were 5.31 ± 0.01 pH units, and 51.6 ± 3.5%, respectively. That degree of protonation is very similar to that obtained by Rinaudo et al. [6,7], who obtained a value around 50%, with no report about the measurement uncertainty. In addition, an acetic acid concentration to chitosan concentration ratio of 0.660 was necessary for solubilization, while Rinaudo et al. used a similar 0.6 ratio. Figure 7 shows the solubility of chitosan as a function of the solvent pH at temperatures between 25 and 50 ºC. The results apparently suggest an exponential increase of the chitosan solubility as solvent pH decrease. However, one would expect an asymptotic tendency at higher chitosan concentration, followed by high viscosity solution that would make difficult its treatment on a process.

pH measurements of water pressurized with carbon dioxide. Figure 8 shows the parity graph of the pH data of pressurized (water + carbon dioxide) system measured in this work (pH_{exp}) and those obtained in other works, at different temperatures. Notice the similarity between the pH data measured in this work with those already reported in the literature, even using different techniques. The minimum and maximum discrepancies between the pH data were 0.01 and 0.08 pH units, respectively. This values are low considering the uncertainty of the measurement, which goes from 0.01 to 0.05 pH units.

Chitosan solubility. The main reactions taking place in these experiments are the following:

Water + carbon dioxide equilibria,

$$H_2O_{(l)} + CO_{2(aq)} \leftrightarrows H_3O^+_{(aq)} + HCO^-_{3\,(aq)} \quad (3)$$

$$HCO^-_{3(aq)} \leftrightarrows H_3O^+_{(aq)} + CO^{-2}_{3\,(aq)} \quad (4)$$

Chitosan equilibria,

$$R-NH_{2(s)} + H_3O^+_{(aq)} \leftrightarrows R-NH_{3\,(aq)}^+ + H_2O_{(l)} \quad (5)$$

Figure 9 shows an example of the chitosan solubilization kinetics in pressurized (water + carbon dioxide). Here, four points are identified: (1) distilled water + indicator + chitosan particles without stirring. (2) the acidification of the water, where reactions (3) and (4) prevail. (3) Chitosan is getting protonated and the reaction (5) prevails, herein chitosan is partially solubilized in the acidified water, notice how the spectrum signal widen. (4) Chitosan solubilization, at this point the system is near the reaction equilibria.

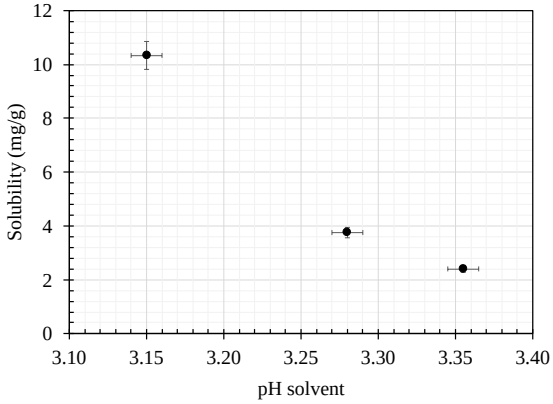

Fig. 7 The Solubility of medium molar mass chitosan like a function of the solvent pH at temperatures between 25 and 50 ºC

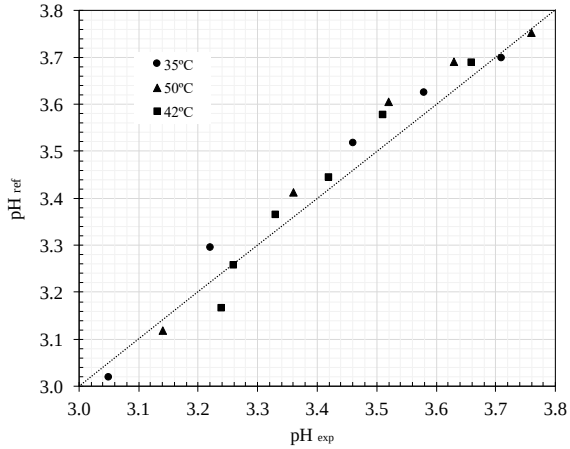

Fig. 8 Parity graph of the pH data of pressurized (water + carbon dioxide) system measured in this work (pH_{exp}) and those obtained in other works [18,19], at 35, 42 and 50 ºC.

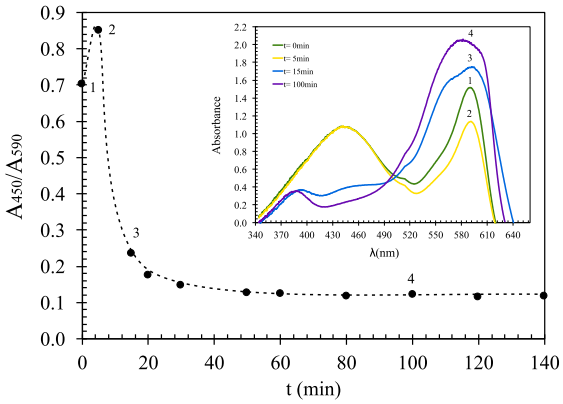

Fig. 9 An example of the chitosan solubilization kinetics in pressurized carbon dioxide and water, at 25 ºC and 500 psi, 2.3(10^{-2}) monoM, *ca.*1500 µm particle size. Dash line only represents a visual tendency.

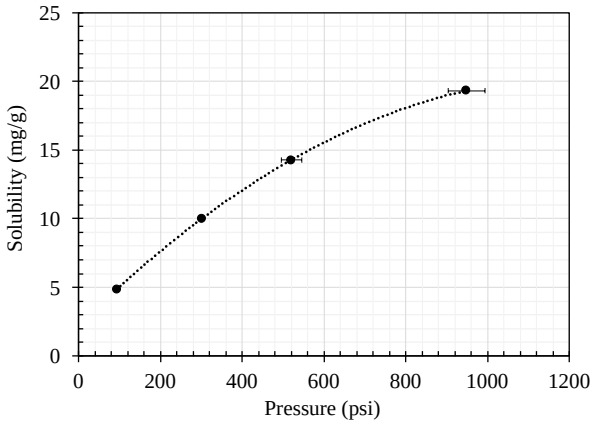

Fig. 10 Solubility of medium molar mass Chitosan in pressurized (water + carbon dioxide) at 25 ºC. Dash line only represents a visual tendency.

Figure 10 shows the solubility of medium molar chitosan in (water + carbon dioxide) system at different pressures. As observed chitosan solubility increases as pressure does, but the behavior tends to be asymptotic. That result makes sense regarding the pH behavior of the pressurized (water + carbon dioxide) system [18,19] , which was validated to be asymptotic. This means that if the initial pH has a limiting value, chitosan solubility will have as well, due to a prone decrease in protonation. However, and as mentioned above, high concentrated chitosan solutions have high viscosity, an important aspect to take into consideration for designing processes and products, which could be overcome increasing the temperature and might be the pressure.

CONCLUSION

Medium-molar-mass chitosan was characterized using the molar mass distribution as determined by SEC and viscosity, and using the degree of acetylation by 1H-NMR and UV measurements. An experimental apparatus for determining solubilities at high pressure was designed and built, and was validated by measuring the degree of protonation at which the solubilization of medium-molar-mass chitosan in aqueous acetic acid occurs, and by determining the pH of the pressurized (water + carbon dioxide) system. Finally, solubility measurements of medium molar mass chitosan in pressurized (water + carbon dioxide) were performed at 25 ºC. An asymptotic increasing behavior of chitosan solubility was observed as pressure increased. The experimental results presented herein will be valuable for designing and developing products using chitosan dissolved in pressurized (water + carbon dioxide), as a green alternative to conventional solvents currently in use.

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