

Document downloaded from:

<http://hdl.handle.net/10251/66730>

This paper must be cited as:

Gamiz Gonzalez, MA.; Piskin, AE.; Pandis, C.; Chatzimanolis-Moustakas, C.; Kyritsis, A.; Marí Soucase, B.; Gómez Ribelles, JL.... (2015). Determining the influence of N-acetylation on water sorption in chitosan films. *Carbohydrate Polymers*. 133:110-116.
doi:10.1016/j.carbpol.2015.07.020.



The final publication is available at

<http://dx.doi.org/10.1016/j.carbpol.2015.07.020>

Copyright Elsevier

Additional Information

Determining the influence of N-acetylation on water sorption in chitosan films

M. A. Gámiz-González^{a,*}, A. E. Piskin^{a,†}, C. Pandis^b, C. Chatzimanolis-Moustakas^b, A. Kyritsis^b, B. Marí^c, J. L. Gómez Ribelles^{a,d}, A. Vidaurre^{a,d}

^a Center for Biomaterials and Tissue Engineering, (CBIT), Universitat Politècnica de València, Cno.de Vera s/n, 46022 Valencia, Spain.

^bPhysics Department, National Technical University of Athens, Greece

^cDepartamento de Física Aplicada-IDF, Universitat Politècnica de València, 46022 Valencia, Spain

^dCenter in Bioengineering, Biomaterials and Nanomedicine of (CIBER-BBN), Valencia, Spain

Research highlights

Water absorption in chitosan increases when the content of acetylated groups increases.

No dependence of the water-polymer interaction on deacetylation degree was found.

No difference in the chain stiffness was found when the samples are immersed in water.

Instability of crystals during swelling process reduced cross-linking density.

It explain the high equilibrium water content of low deacetylation degree chitosans.

Keywords

Chitosan, Deacetylation degree, Water absorption, Flory Huggins parameter, Chain stiffness, Crystallinity.

*Corresponding author. Tel.: +34 96 387 007; Fax: +34 96 387 276.

E-mail address: agamiz@upvnet.upv.es

[†] Permanent address: Department of polymer Science and Technology, Faculty of Science and Letters, Istanbul Technical University, 34469, Maslak, Istanbul, Turkey.

1

2 **Abstract**

3 Water absorption in chitosan rapidly increases when the deacetylation degree
4 decreases between 85 and 45%. This seems to contradict the fact that water
5 absorption in chitin is much lower than that of chitosan. The aim of this paper is to
6 understand this feature by measuring the main parameters affecting equilibrium
7 water content. Since swelling capacity depends on the water-polymer interaction, the
8 Flory Huggins interaction parameter was evaluated, finding small or null dependence
9 on the deacetylation degree. Other factor influencing elastic energy is chain stiffness
10 related to the elastic modulus that was measured as a function of deacetylation
11 degree. Besides, crystalline structure was measured by X ray diffraction patterns as a
12 characteristic of cross-linking density. These observations led us to conclude that the
13 instability of crystals during the swelling process increases with decreasing
14 deacetylation degree, explaining the high equilibrium water content of low
15 deacetylation chitosans.

16 **1.Introduction**

17 Chitin is the second most abundant natural polysaccharide next to cellulose. It is
18 obtained from natural resources like insects, marine invertebrates and fungi. The N-
19 deacetylation degree is the relationship between N-acetyl groups (-NHCOCH₃), with
20 respect to the total amount of N-acetyl and amine (-NH₂) groups in the polymer.
21 Chitin is a fully acetylated polymer and is called *chitosan* when the deacetylation
22 degree (DD) is between 50-90%, but these limits are arbitrary (Baldrick, 2010).

23 Chitosan is thus a copolymer formed by glucosamine and N-acetyl glucosamine units
24 linked by O-glycosidic bonds $\beta(1\rightarrow4)$ and is a biocompatible, biodegradable and non-
25 toxic hydrogel (Bhattarai, Gunn, & Zhang, 2010). Due to its natural origin and
26 physical properties, it is one of the hydrogels proposed for many applications in the
27 field of tissue engineering, regenerative medicine, pharmaceuticals, in particular drug
28 delivery, and the cosmetic industry (Dash, Chiellini, Ottenbrite, & Chiellini, 2011),
29 (Ravi Kumar, 2000).

30 The physicochemical properties of chitosan vary widely and depend on factors such
31 as the natural source from which the polymer is obtained, or the chemical process

1 yielding N-acetylation or/and N-deacetylation to obtain the desired DD value (Jolanta
2 Kumirska, Weinhold, Thöming, & Stepnowski, 2011). The N-deacetylation degree
3 (DD) is one of the most important determinants of chitosan behavior concerning
4 physicochemical properties such as reactivity (Sashiwa & Shigemasa, 1999),
5 crystallinity (Samuels, 1981), swelling degree and mechanical properties (Mano,
6 2008; Mucha & Pawlak, 2005). Chitosan exhibits a highly hydrophilic behavior
7 (Ludwiczak & Mucha, 2010) due to the presence of hydroxyl and amino groups. The
8 complete removal of the water molecules upon drying, even at temperatures above
9 100°C, is very difficult (Viciosa, Dionísio, & Mano, 2006).

10 In this work chitosan with varying DD was prepared in mild conditions by acetylation
11 reaction (Hirano, Ohe, & Ono, 1976) of high DD medical grade chitosan with acetic
12 anhydride. It was found that the equilibrium water content increases when DD
13 decreases. The aim of this work is to get a better understanding of this behavior, so
14 important in tissue engineering applications. To the best of our knowledge this paper
15 is the first experimental study determining the main parameters of the equilibrium
16 water content of chitosan with varying DD in a homologous series of samples.

17 **2. Materials and Methods**

18 **2.1 Materials**

19 Chitosan, medical grade polymer, was purchased from Novamatrix (Protasan UP B
20 80/20) with 80-89% degree of deacetylation, according to the supplier's information,
21 and apparent viscosity of 20-199 mPa.s. Acid acetic, acetic anhydride, deuterium
22 oxide (D₂O) and deuterium chloride (DCI), analytical grade, were purchased from
23 Sigma-Aldrich and used without further purification. Sodium iodide and sodium
24 hydroxide pellets were purchased from Scharlau and used as received.

25 **2.2 N-Acetylation reaction**

26 Chitosan was dissolved in acid acetic 2 % (w/w) under stirring for three hours, after
27 which methanol was added and the solution was left to stir overnight. Different
28 quantities of acetic anhydride were mixed with methanol and were added to the
29 chitosan solution (Hirano et al., 1976). The reaction was stirred overnight and then
30 NaOH 1M was added to neutralize the chitosan solution. All synthesis steps were
31 carried out at room temperature.

1 The chitosan samples were washed several times with water until neutral pH was
2 obtained. Chitosan was freeze-dried to obtain a white powder with different
3 deacetylation degrees.

4 **2.3 ¹H-NMR Spectroscopy**

5 The deacetylation degree (DD) was determined by nuclear magnetic resonance of
6 proton spectroscopy (¹H-NMR) using a Varian Unity Plus 300 (Fernandez-Megia,
7 Novoa-Carballal, Quiñoá, & Riguera, 2005; Lavertu et al., 2003). Five milligrams of
8 the synthesized chitosan powder were added to NMR tubes containing 1 mL of 2%
9 (v/v) deuterium chloride solution in deuterated water. The chitosan solutions were
10 heated to 70°C for 2-3 hours, carrying out the experiment at this temperature. The
11 deacetylation degree of the samples was calculated by the equation (Lavertu et al.,
12 2003):

$$DD(\%) = \left(\frac{H1D}{H1D + HAc/3} \right) 100 \quad (1)$$

13

14 Where H1D is the signal area corresponding to the proton from C1 of glucosamine
15 unit and HAc is the signal area corresponding to the proton from methyl group of N-
16 Acetyl-glucosamine unit.

17 **2.4 Gel permeation chromatography**

18 Molecular weight distribution of chitosan films with different DD were analyzed using
19 a Gel Permeation Chromatographer, GPC, at 35 °C using a Waters Breeze GPC
20 system with a 1525 Binary HPLC pump (Waters Corporation, Milford, MA) equipped
21 with a 2414 refractive index detector and four serial columns of water (Ultrahydrogel
22 7.8 mm ID x 30 cm). The samples were dissolved in a buffer solution of CH₃COOH
23 0.5M/CH₃COONa 0.2M, pH=4.5 that was also used as mobile phase at a flow rate of
24 0.5 ml/min and 20µl injection volume. The calibration curve was prepared by using
25 monodisperse PEO (polyethylene glycol) standards (Showa denko).

26

1 **2.5 Preparation of chitosan films**

2 Chitosan films were formed from a 2 % (w/v) solution by a solvent casting method
3 (Kim, Son, Kim, Weller, & Hanna, 2006). Chitosan solutions with different DD were
4 prepared in an aqueous solution of acetic acid 2% (v/v). When the chitosan solution
5 was totally dissolved, it was filtered through a nylon cloth to remove any insoluble
6 fractions and put in a teflon mold until the solvent was completely evaporated. After
7 drying, the films were neutralized in 25ml (NaOH) sodium hydroxide 1M aqueous
8 solution in order to deprotonate the amine groups of chitosan chains. Finally, the
9 films obtained were washed several times with distilled water until neutral pH.

10

11 **2.6 Equilibrium water content**

12 Chitosan films (n=5 replicates for each DD) were dried in vacuum desiccators at
13 50°C and weighed (W_0). They were then immersed in deionized water at room
14 temperature until equilibrium (72 hours). The swollen films were taken out of the
15 liquid water and their surfaces were wiped with filter paper to remove excess water
16 and then weighed, W_s .

17 The equilibrium water content for each sample was calculated from the following
18 equation:

$$Q_{eq}(\%) = \frac{W_s - W_0}{W_0} 100 \quad (2)$$

19

20 **2.7 Water vapor absorption isotherms**

21 Water vapor absorption experiments were performed by the gravimetric technique at
22 25°C using a VTI-SA+ (TA Instruments) vapor absorption analyzer for water activities
23 up to 0.95. The water weight content, h , is defined as:

$$h = \frac{m_{water}}{m_{dry\ polymer}} \quad (3)$$

24 The volume fraction of water was calculated from the following relation:

$$\phi = \frac{h}{h + \rho_w / \rho_{dry}} \quad (4)$$

1 Where ρ_{dry} and ρ_{w} are polymer and water density respectively.

2 The density of the polymer samples (ρ_{dry}) was determined using the Density Kit
3 mounted to a Mettler Toledo (AX 205) balance. The measurements for density
4 determination were performed based on Archimedes' principle by weighting the dried
5 samples in air and when immersed in an auxiliary liquid of known density. N-octane
6 (purchased from Sigma-Aldrich) was used as the auxiliary liquid because chitosan
7 does not dissolve or swell in it.

8

9 **2.8 Crystallinity**

10 X-ray diffraction spectra of the chitosan films were obtained on a Rigaku Ultima IV X-
11 ray diffractometer in the Bragg-Bentano configuration using the $K\alpha$ radiation of a Cu
12 anode. The dry samples were scanned from $2\theta=5-70^\circ$ at a speed of $2^\circ/\text{min}$.
13 Diffraction patterns were deconvoluted, using "PDXL" Integrated X-Ray powder
14 diffraction software, to three non symmetrical Gaussian peaks. Relative crystallinity
15 was determined as the ratio to the integrated crystalline intensity to the total intensity
16 for comparative purposes (J. Kumirska et al., 2010). Diffraction spectra were
17 measured for wet samples from $2\theta=5-35^\circ$ at a speed of $2^\circ/\text{min}$. Wet samples were
18 immersed in deionized water until they were placed in the diffractometer chamber.

19 **2.9 Mechanical testing**

20 Dynamical mechanical analysis of the CHT films was carried out by a tensile test in a
21 Perkin Elmer DMA 8000 device. Five samples ($n=5$) of each chitosan film were cut
22 into long narrow strips with a cross section of approximately 5 mm in width.
23 Measurements were performed on samples of 6 mm free length, at room
24 temperature, 1 Hz, and at different levels of relative humidity in both swollen and dry
25 states. The samples were equilibrated in sealed desiccators containing over
26 saturated salt solutions of NaCl, NaI and pure water, to obtain relative humidities of
27 36%, 75% and 100%, respectively. The samples were then mounted in the device
28 and a flow of air with the same %RH was allowed to circulate through the measuring
29 chamber. This humid air was obtained by allowing air to bubble in three consecutive
30 recipients containing the corresponding over saturated salt solutions (NaCl, NaI and
31 water). The measurements were also performed on dry samples and on samples
32 previously immersed in liquid water. These samples were maintained immersed in

1 deionized water, placed in a teflon reservoir, while the measurements took place. In
2 all cases the samples were allowed to equilibrate before the dates were recorded.

3 2.10. Statistical analysis

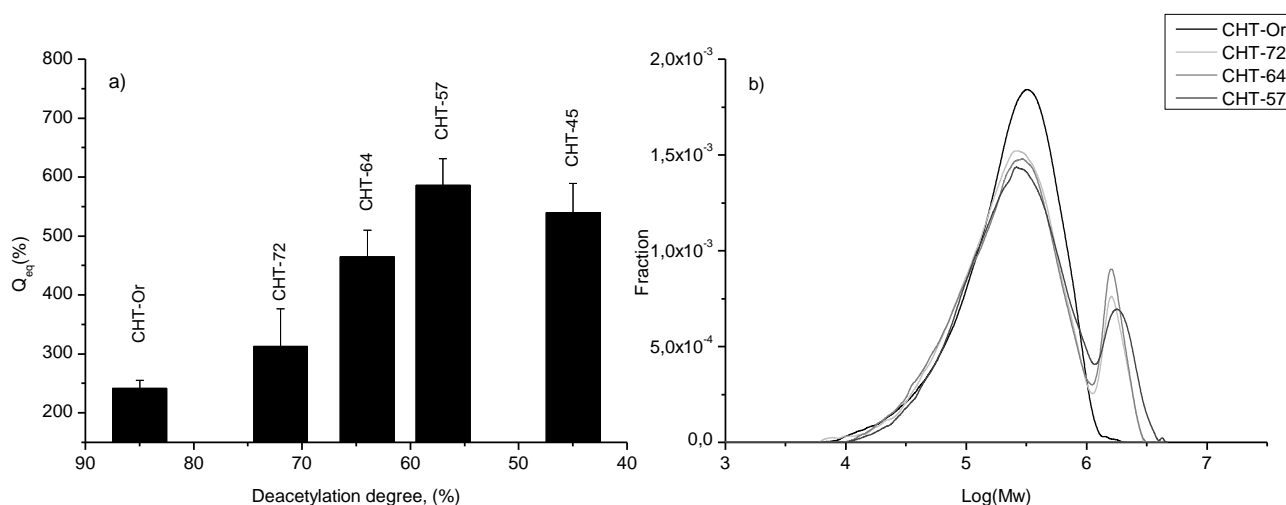
4 The data were analyzed by ANOVA using an SPSS computer program (SPSS
5 Statistic 16.0), while means were compared by the Tukey-b test with the level of
6 significance set at $P < 0.05$.

8 3. Results and discussions

9 Chitosan with different deacetylation degrees (DD) ranging from values of 72 to 45 %
10 were obtained from CHT-Or with DD=85%, as determined by $^1\text{H-NMR}$ spectroscopy,
11 following Eq (1). GPC measurements indicated (Fig 1b) an increase in molecular
12 weight with decreasing deacetylation, that suggest the formation of aggregates of
13 higher molecular weight (Chen, Du, Tian, & Sun, 2005). The samples are given a
14 number after the CHT to indicate their DD, for example, CHT-45 means DD=45%.

15 3.1 Equilibrium water content. Water absorption from the liquid.

16 Figure 1 shows that the equilibrium water content (Q_{eq}) increases when the
17 deacetylation degree decreases. The Q_{eq} for CHT-Or is around $242 \pm 13\%$ whereas it
18 is $540 \pm 50\%$ for CHT-45. This result is in good agreement with the literature (Bagheri-
19 Khoulenjani, Taghizadeh, & Mirzadeh, 2009). Statistical analysis showed that there is
20 no significant difference in Q_{eq} between samples with lower DDs (CHT-64, CHT-57
21 and CHT-45).



1 Figure 1.a) Equilibrium water content (Q_{eq}) of chitosan with different deacetylation degrees
2 after 72 hours immersed in deionizer water at room temperature. b) Molecular weight
3 distribution of chitosan with varying deacetylation degrees.

4
5 Chitosan is water soluble at acidic pH but, as can be seen in Fig.1, at pH7, non-
6 protonated chitosan is able to absorb really large amounts of water but it is not water
7 soluble. This can be explained by the formation of a network structure in which
8 crystalline entities and physical entanglements play the role of physical crosslinks
9 even when chitosan was not chemically cross-linked. Thus, swelling behavior must
10 be analyzed in terms of the swelling theory of polymer networks. The Flory-Rehner
11 equation is obtained by minimizing the free energy of the swollen network, which is
12 the sum of the free energy of mixing (determined by Flory interaction parameter
13 between the polymer segments and the solvent) and the elastic free energy
14 depending on the length and stiffness of the polymer chains between cross-links. The
15 Flory-Rehner equation (Erman & Mark, 1997; Flory, 1944) gives the relationship
16 between the swelling capacity of a polymer network when immersed in a liquid
17 solvent and the number of effective polymer chains between crosslinks per unit
18 volume of polymer, n_c/V

$$\ln(\phi) + (1 - \phi) + \chi(1 - \phi)^2 + v_{sol} \frac{n_c}{V} (1 - \phi)^{\frac{1}{3}} = 0 \quad (5)$$

19 where ϕ is the volume fraction of water in the swollen network, v_{sol} is the molar
20 volume of the solvent and χ is the Flory interaction parameter between polymer and
21 solvent and n_c/V will be determined by the number of crystalline structures or chain
22 entanglements.

23 From equation (5) one can infer that the increase in water content when DD
24 decreases, as shown in Fig 1, can only be explained by (i) a decrease of χ and /or (ii)
25 a decrease of chain stiffness and/or (iii) a decrease of crystallinity when DD falls to
26 between 85 and 50%.

27 **3.2 Water absorption from the vapor phase: Flory Huggins parameter**

28

29 In order to verify the influence of the polymer-water interaction, the absorption
30 isotherms were analyzed by measuring the water content of chitosan films

1 equilibrated in a vapor atmosphere at different relative humidities, i.e. with varying
 2 water activity a_w . Figure 2 shows the water content measured on a dry basis, h , as a
 3 function of water activity. These isotherms belong to Class II in the Brunauer
 4 classification (Brunauer, Deming, Deming, & Teller, 1940). The above sigmoidal
 5 shape is typical of hydrogels, while the departure from linear behavior for higher
 6 water activities is explained in terms of the clustering of water molecules (George L,
 7 1980).

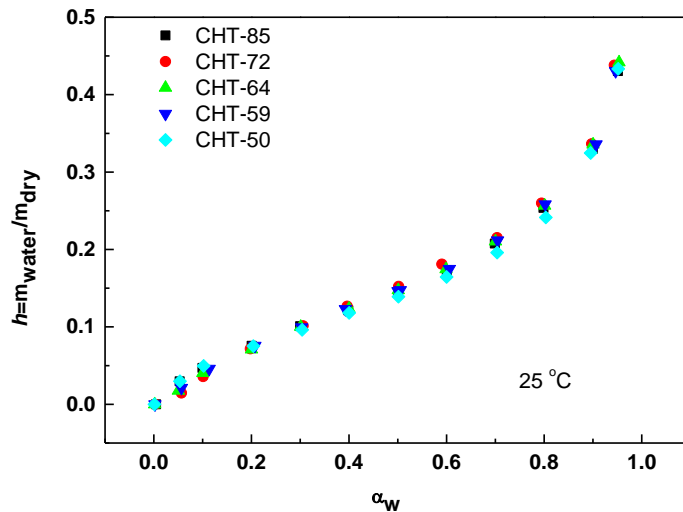
8 The water-polymer interaction parameter was calculated from the Flory-Huggins
 9 theory (Flory PJ, 1953), (Pradas et al., 2001), by the equation:

$$\ln a_w = \ln \phi + (1 - \phi) + \chi(1 - \phi)^2 \quad (6)$$

10 Where, a_w is the water activity, and ϕ and χ have been previously described.

11

12



13

14 Figure 2. Water content in the films with different DD to different water activity.

15

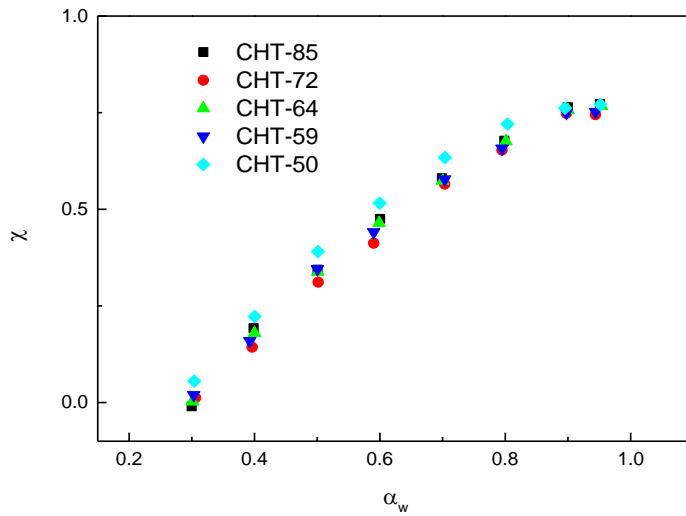
16 It should be emphasized the great difference between the amount of water adsorbed
 17 from the vapor phase with that from liquid water. The water uptake obtained from
 18 measurements of water vapor absorption is about 45% on dry basis, for the highest
 19 water activity, that is $a_w=0.95$. In contrary, when immersed in liquid water the water
 20 content varies between 250-600%. Similar high values have been reported

1 previously (Pandis et al., 2014) for chitosan films. The above behavior is typical for
2 hydrogels and could be explained by taking into consideration that the amount of
3 water absorbed in the form of bulk water is much higher than that forming a
4 homogeneous mixed phase with the hydrogel (Pandis et al., 2011). The former
5 depends more on the elasticity of the network, whereas the latter depends more on
6 the number of absorption sites in the polymer chains. Furthermore the significant
7 network expansion of chitosan when immersed in liquid water could also be followed
8 by the disentanglement of micelles formed by hydrophilic groups (hydroxyl and amino
9 groups) , creating in that way new absorption sites and additional space for lodging of
10 liquid water (Monleon Pradas, Salmeron Sanchez, Gallego Ferrer, & Gomez Ribelles,
11 2004).Consequently, the equilibrium water content is appreciably higher when
12 compared with absorption from water vapor.

13

14 The apparent interaction parameter χ was calculated from the data obtained from the
15 water vapor absorption isotherms using Eq.(5) and Eq.(6). As water absorption
16 isotherms are practically independent of DD, the interaction parameter is also
17 independent of DD. Figure 3 shows the Flory Huggins interaction parameters
18 calculated between $0.3 < a_w < 1$, (when water activity was lower than 0.3, samples were
19 not able to reach equilibrium during the experimental time). The χ values increase
20 with water activity, reaching a value around 0.7 for all samples at the highest relative
21 humidity level. It is worth highlighting here that the affinity of chitosan to water
22 molecules does not significantly depend on DD.

23



1

2 Figure 3. Flory Huggins interaction parameter of chitosan films as a function of water activity
 3 in the vapor phase.

4

5 This result is in agreement with what could be obtained from group contribution
 6 calculations. This method can be estimated from solubility parameters of solvent, δ_s ,
 7 and polymer, δ_p , (Van Krevelen & Te Nijenhuis, 2009):

$$\chi_{ps} = 0.34 + \frac{v_s}{RT} (\delta_p - \delta_s)^2 \quad (7)$$

8 For the different deacetylation degrees, the solubility parameter of the polymer can
 9 be calculated from that of chitin and chitosan by:

$$\delta_p = (\delta_{chitin} \left(1 - \frac{DD}{100}\right) + \delta_{chitosan} \frac{DD}{100}) \quad (8)$$

10 The solubility parameter values of chitin and 100% deacetylated chitosan are not
 11 significantly different. The average of the values determined by different group
 12 contribution methods was $\delta_{chitin}=41.15 \text{ J}^{1/2}\text{cm}^{-3/2}$ while $\delta_{chitosan}=43.06 \text{ J}^{1/2}\text{cm}^{-3/2}$
 13 (Ravindra, Krovvidi, & Khan, 1998).

14 The interaction parameter of 0.55 for CHT-Or and 0.58 for CHT-59 was obtained by
 15 applying Eqs.(7) and (8).

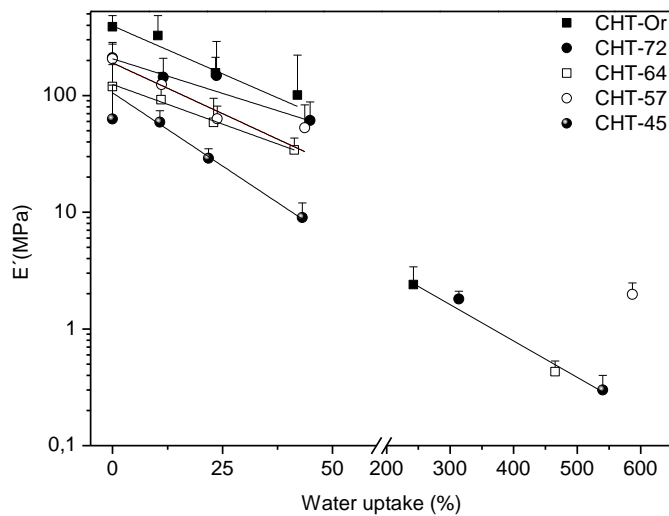
16 3.3 Mechanical testing

17 The mechanical tests on the different chitosan films were analyzed in order to
 18 determine the effect of chain stiffness on the swelling capacity of the polymer. The

1 experiments were carried out at room temperature under five different conditions: in a
2 dry state in a vapor atmosphere, at three different relative humidities, and immersed
3 in liquid water.

4 As could be expected, the elastic modulus shown in Fig.4 falls as water content of
5 the sample rises, both in a vapor atmosphere and immersed in liquid water, and the
6 elastic modulus also increases with DD when the samples are measured in a vapor
7 atmosphere. This indicates that the stiffness of the polymer chain increases with the
8 presence of amine groups in the backbone of the polymer. However, when the
9 samples are immersed in water, their elastic modulus seems to depend on water
10 content, whatever the DD. The straight line in Figure 4 for water content above 200%
11 is intended to be a guide only.

12 It is accepted (Dumitriu, 2001) that the differences in the conformational mobility of
13 the chitosan chains with DD can be affected by the highly packed chains due to inter-
14 chain interactions, mainly by hydrogen bonding; and these interactions are favored
15 when DD is reduced. Nevertheless, the results shown in Figure 4 seem to indicate
16 that, as the polymer is immersed in water, the rearrangements of the chains are quite
17 similar for all DDs, so that chain stiffness could not be the determinant parameter for
18 water content. The dependence of chain stiffness with DD for lower water contents
19 could produce a change in the absorption kinetics, since the higher chain stiffness in
20 the more deacetylated samples could slow down the absorption rate in the first
21 stages of the absorption process, while equilibrium final states could be unaffected.
22 This hypothesis was nevertheless not checked, as being outside the scope of this
23 work.



1

2 Figure 4. Elastic modulus of chitosan films at room temperature with different deacetylation
 3 degrees in a dry state, different relative humidity conditions, and immersed in water. Lines
 4 are only intended as guides.

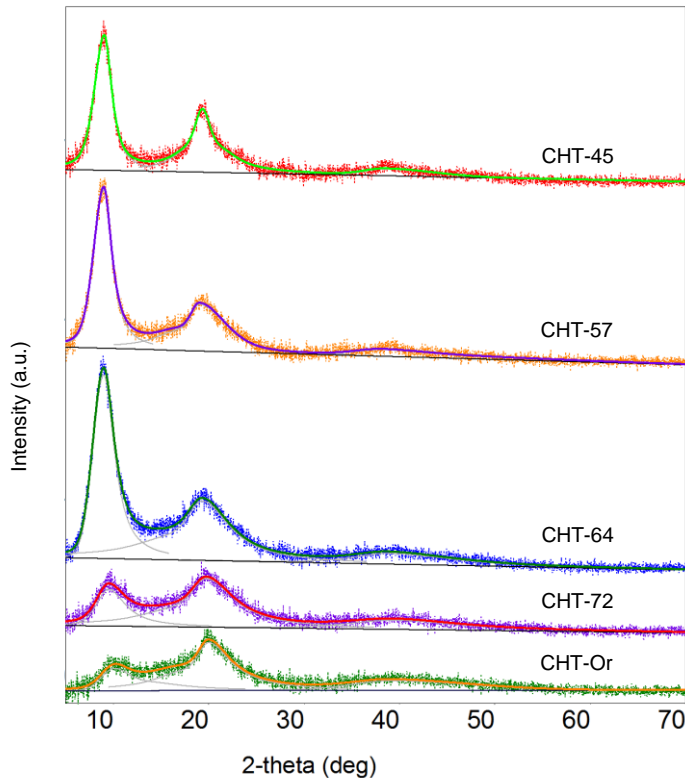
5

6 3.4 Crystallinity

7 If the large increase in gel swelling capacity with decreasing deacetylation cannot be
 8 ascribed either to the polymer-water interaction or to chain stiffness, the parameter
 9 that determines this behavior can only be the density of the physical crosslinks
 10 (crystals).

11 In order to verify this hypothesis, the X ray diffraction spectra were measured. Fig. 5
 12 shows the diffractograms of samples in a dry state. Diffraction peaks were
 13 deconvoluted using three non symmetrical Gaussian peaks showing a typical
 14 spectrum of the chitosan polymer with two strong reflections at $2\theta=9-10^\circ$ for (020)
 15 reflections and at $2\theta=20-21^\circ$ for (110) and (120) reflections (Jolanta Kumirska et al.,
 16 2011), (Jaworska, Sakurai, Gaudon, & Guibal, 2003). The wide peak, located about
 17 38° , with crystallite size lower than 9 \AA is ascribed to the amorphous (Kobaisi,
 18 Murugaraj, & Mainwaring, 2012). The films with the highest DDs present the most
 19 sharp and intense peaks around $2\theta=20-21^\circ$, whereas the films with lower DDs show
 20 the most intense peaks around $2\theta=9-10^\circ$. Relative crystallinity, calculated by dividing
 21 the area of the crystalline peaks by the total area under the curve, increases as DD
 22 decreases, ranging from $79\% \pm 2\%$ for CHT-Or to $92.6\% \pm 0.6\%$ for CHT-45 (Jaworska

1 et al., 2003). This change in crystallinity is attributed to the incorporation of different
2 amounts of acetyl groups in the polymer, consequently reorganizing the chitosan's
3 crystalline structure.



4

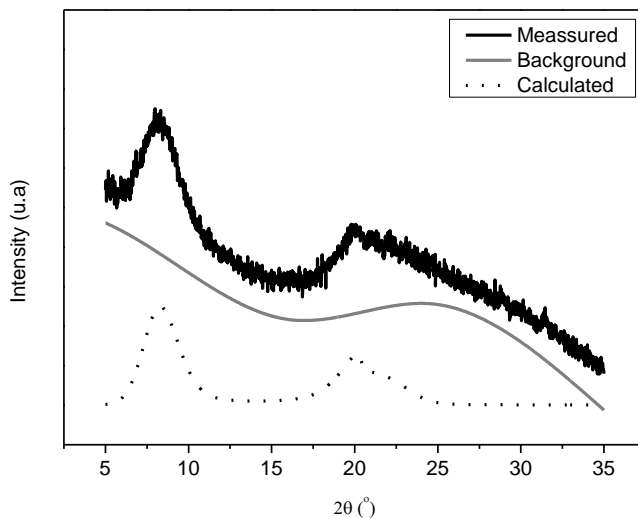
5 Figure 5. Diffractograms of chitosan films with different DDs measured in a dry state. The
6 experimental spectra, the Gaussians for each peak(grey), and the model obtained by
7 gaussians superposition are shown in the plot.

8

9 Water also plays an important role in the formation of chitosan's crystal structure, as
10 the peak observed at 10° is generally attributed to the integration of water molecules
11 in the crystal lattice (Okuyama, Noguchi, Miyazawa, Yui, & Ogawa, 1997). More
12 specifically, this peak is associated with the linkage between acetylated glucosamine
13 (GlcNAc) groups of neighboring chains, facilitating the incorporation of water
14 molecules into such a network and forming a hydrated crystal (Kittur, Vishu Kumar, &
15 Tharanathan, 2003). Therefore, while it is logical to see the lower intensity in the XRD
16 diffractograms in the samples with higher DDs, meaning fewer acetylated
17 glucosamine units, on the other hand, it is worth noting that the intensity of that peak
18 reaches the maximum intensity for the sample with 64% DD, implying that the large

1 amount of glycosamine groups may cause steric hindrance to the formation of this
2 hydrated crystal structure.

3 In order to verify the relationship between crystalline structure and water absorption
4 capacity, the X-ray diffraction spectra of samples with different water contents were
5 measured. The experimental procedure was as follows: swollen samples in water
6 were placed in the diffractometer and spectra were collected while the samples were
7 losing water. The diffractogram of the hydrated sample was obtained by the
8 difference between the measured diffractogram and the background, which includes
9 the water signal. Fig 6 shows an example of the diffractogram of a swollen sample,
10 the background, and the difference between both signals. A new scan was measured
11 every 3 minutes until no further changes were observed.

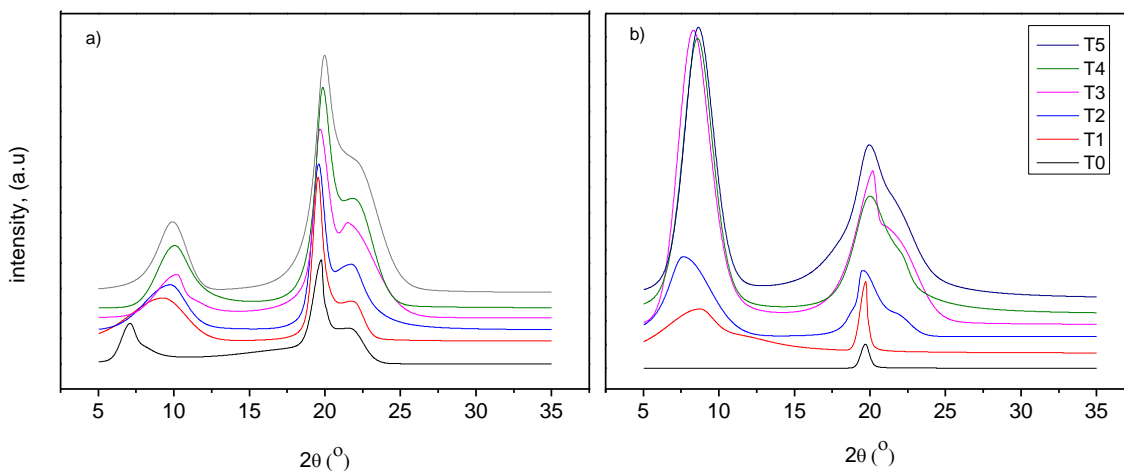


12

13 Figure 6. Diffractograms of a swollen sample, background and the difference between both
14 signals, considered as the diffractogram of the hydrated sample.

15

16 Fig 7 shows the calculated diffractograms for CHT-Or and CHT-64 as a function of
17 time. It can be observed that significant changes in the crystalline structure occur
18 during the drying process. In all cases the dry samples (T5) show more intense
19 peaks than the corresponding swollen samples (T0).



1
2 Figure 7. Calculated diffractograms of a) CHT-Or and b) CHT-64 taken every 3 minutes
3 (T0=0 min, T1=3 min and so on) starting from swollen films until dry at room temperature.

4
5 The most deacetylated sample, CHT-Or, shows comparatively fewer changes with
6 water content than the most acetylated ones. For the CHT-Or sample, small changes
7 were observed in the $2\theta=10^\circ$ peak, whereas an increase in the intensity of the
8 $2\theta=20^\circ$ peak is observed throughout the drying process. In contrast, significant
9 changes were observed in the rest of the samples; e.g. Fig 7b shows the results for
10 CHT-64. It can be observed that the crystalline peak around $2\theta=9-10^\circ$ does not
11 appear in the swollen state and the $2\theta=20^\circ$ peak presents a very low intensity. These
12 results suggest that water dissolves part of the crystals and that this effect is more
13 pronounced in samples with lower DD. Both crystalline peaks increase in intensity as
14 the film loses water. This behavior was also observed by (Rinaudo, 2006), who
15 stated that the β -chitin shows a reversible crystalline structure when water molecules
16 disappear from inside the sample.

17 To sum up, the DRX results show that the samples with higher DDs present a more
18 stable crystalline structure, which acts as a crosslinker, hindering the expansion of
19 the network. On the other, the samples with the lowest DDs, which are more
20 crystalline in the dry state, become less crystalline in the swollen state due to the
21 dissolution of a part of their crystals.

22

1 **Conclusions**

2 In this paper we analyzed the physical origin of the increase of chitosan's equilibrium
3 water content with decreasing degrees of deacetylation. Three different physical
4 properties were studied: the interaction water-polymer parameter χ , the elasticity of
5 the polymer chain, and the crystalline structure.

6 The results show that the low dependence of the polymer-water interaction
7 parameter on the degree of deacetylation cannot explain the differences in
8 equilibrium water content. The mechanical test indicates some differences in polymer
9 chain stiffness when the samples contain small amounts of water (from the vapor
10 atmosphere), but no differences when they are immersed in water. The differences in
11 chain stiffness with deacetylation degree cannot therefore explain the evolution of the
12 water absorption behavior either. However, the DRX diffractograms show significant
13 differences in the crystalline structure of the dry and swollen samples. In the dry state
14 those with lowest DD are more crystalline than CHT-Or. However, when the samples
15 are immersed in water the tendency changes and those with the highest DD are the
16 most crystalline.

17 In conclusion, this study of the crystalline structure of swollen samples finds that
18 equilibrium water content increases as DD decreases, due to the crystalline regions
19 which act as physical crosslinkers and are partially dissolved by water. This effect is
20 more pronounced in the samples with lower DD.

21 **Acknowledgements**

22 The authors gratefully acknowledge the financial support from the Spanish Ministry of
23 Economy and Competitiveness through the MAT2013-46467-C4-1-R project,
24 including FEDER funds. CIBER-BBN is an initiative funded by the VI National R&D&I
25 Plan 2008-2011, *Iniciativa Ingenio 2010*, *Consolider Program*, CIBER Actions and
26 financed by the *Instituto de Salud Carlos III* with the assistance of the European
27 Regional Development Fund.

28

29

30

1 References

- 2 Bagheri-Khoulenjani, S., Taghizadeh, S. M., & Mirzadeh, H. (2009). An investigation on the short-term
3 biodegradability of chitosan with various molecular weights and degrees of deacetylation.
4 *Carbohydrate Polymers*, 78(4), 773-778. doi: <http://dx.doi.org/10.1016/j.carbpol.2009.06.020>
- 5 Baldrick, Paul. (2010). The safety of chitosan as a pharmaceutical excipient. *Regulatory Toxicology*
6 *and Pharmacology*, 56(3), 290-299. doi: <http://dx.doi.org/10.1016/j.yrtph.2009.09.015>
- 7 Bhattarai, Narayan, Gunn, Jonathan, & Zhang, Miqin. (2010). Chitosan-based hydrogels for
8 controlled, localized drug delivery. *Advanced Drug Delivery Reviews*, 62(1), 83-99. doi:
9 <http://dx.doi.org/10.1016/j.addr.2009.07.019>
- 10 Brunauer, Stephen, Deming, Lola S., Deming, W. Edwards, & Teller, Edward. (1940). On a Theory of
11 the van der Waals Adsorption of Gases. *Journal of the American Chemical Society*, 62(7),
12 1723-1732. doi: 10.1021/ja01864a025
- 13 Chen, Lingyun, Du, Yumin, Tian, Zhigang, & Sun, Liping. (2005). Effect of the degree of deacetylation
14 and the substitution of carboxymethyl chitosan on its aggregation behavior. *Journal of*
15 *Polymer Science Part B: Polymer Physics*, 43(3), 296-305.
- 16 Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan—A versatile semi-synthetic
17 polymer in biomedical applications. *Progress in Polymer Science*, 36(8), 981-1014. doi:
18 <http://dx.doi.org/10.1016/j.progpolymsci.2011.02.001>
- 19 Dumitriu, Severian. (2001). *Polymeric biomaterials, revised and expanded*: CRC Press.
- 20 Erman, Burak, & Mark, James E. (1997). *Structures and properties of rubberlike networks*: Oxford
21 University Press New York.
- 22 Fernandez-Megia, Eduardo, Novoa-Carballal, Ramón, Quiñoá, Emilio, & Riguera, Ricardo. (2005).
23 Optimal routine conditions for the determination of the degree of acetylation of chitosan by
24 ¹H-NMR. *Carbohydrate Polymers*, 61(2), 155-161. doi:
25 <http://dx.doi.org/10.1016/j.carbpol.2005.04.006>
- 26 Flory, Paul J. (1944). Network Structure and the Elastic Properties of Vulcanized Rubber. *Chemical*
27 *reviews*, 35(1), 51-75.
- 28 George L. Brown. (1980). Clustering of Water in Polymers *Water in Polymers* (Vol. 127, pp. 441-450):
29 AMERICAN CHEMICAL SOCIETY.
- 30 Hirano, Shigehiro, Ohe, Yasuo, & Ono, Haruhiro. (1976). Selective N-acylation of chitosan.
31 *Carbohydrate Research*, 47(2), 315-320. doi: <http://dx.doi.org/10.1016/S0008->
32 [6215\(00\)84198-1](http://dx.doi.org/10.1016/S0008-6215(00)84198-1)
- 33 Jaworska, Malgorzata, Sakurai, Kensuke, Gaudon, Pierre, & Guibal, Eric. (2003). Influence of chitosan
34 characteristics on polymer properties. I: Crystallographic properties. *Polymer International*,
35 52(2), 198-205. doi: 10.1002/pi.1159
- 36 Kim, Ki Myong, Son, Jeong Hwa, Kim, Sung-Koo, Weller, Curtis L., & Hanna, Milford A. (2006).
37 Properties of Chitosan Films as a Function of pH and Solvent Type. *Journal of Food Science*,
38 71(3), E119-E124. doi: 10.1111/j.1365-2621.2006.tb15624.x
- 39 Kittur, F. S., Vishu Kumar, A. B., & Tharanathan, R. N. (2003). Low molecular weight chitosans -
40 Preparation by depolymerization with *Aspergillus niger* pectinase, and characterization.
41 *Carbohydrate Research*, 338(12), 1283-1290.
- 42 Kobaisi, Mohammad Al, Murugaraj, Pandiyan, & Mainwaring, David E. (2012). Origin and influence of
43 water-induced chain relaxation phenomena in chitosan biopolymers. *Journal of Polymer*
44 *Science Part B: Polymer Physics*, 50(6), 403-414. doi: 10.1002/polb.23023
- 45 Kumirska, J., Czerwicka, M., Kaczynski, Z., Bychowska, A., Brzozowski, K., Thoming, J., & Stepnowski,
46 P. (2010). Application of Spectroscopic Methods for Structural Analysis of Chitin and
47 Chitosan. *Marine Drugs*, 8(5), 1567-1636. doi: 10.3390/md8051567
- 48 Kumirska, Jolanta, Weinhold, Mirko X., Thöming, Jorg, & Stepnowski, Piotr. (2011). Biomedical
49 Activity of Chitin/Chitosan Based Materials—Influence of Physicochemical Properties Apart
50 from Molecular Weight and Degree of N-Acetylation. *Polymers*, 3(4), 1875-1901.

- 1 Lavertu, M., Xia, Z., Serreji, A. N., Berrada, M., Rodrigues, A., Wang, D., . . . Gupta, Ajay. (2003). A
2 validated ¹H NMR method for the determination of the degree of deacetylation of chitosan.
3 *Journal of Pharmaceutical and Biomedical Analysis*, 32(6), 1149-1158. doi:
4 [http://dx.doi.org/10.1016/S0731-7085\(03\)00155-9](http://dx.doi.org/10.1016/S0731-7085(03)00155-9)
- 5 Ludwiczak, S., & Mucha, M. (2010). Modeling of water sorption isotherms of chitosan blends.
6 *Carbohydrate Polymers*, 79(1), 34-39. doi: <http://dx.doi.org/10.1016/j.carbpol.2009.07.014>
- 7 Mano, João F. (2008). Viscoelastic Properties of Chitosan with Different Hydration Degrees as Studied
8 by Dynamic Mechanical Analysis. *Macromolecular Bioscience*, 8(1), 69-76. doi:
9 10.1002/mabi.200700139
- 10 Monleon Pradas, Manuel, Salmeron Sanchez, Manuel, Gallego Ferrer, Gloria, & Gomez Ribelles, Jose
11 Luis. (2004). Thermodynamics and statistical mechanics of multilayer adsorption. *The Journal*
12 *of chemical physics*, 121(17), 8524-8531.
- 13 Mucha, Maria, & Pawlak, Adam. (2005). Thermal analysis of chitosan and its blends. *Thermochimica*
14 *Acta*, 427(1-2), 69-76. doi: <http://dx.doi.org/10.1016/j.tca.2004.08.014>
- 15 Okuyama, K., Noguchi, K., Miyazawa, T., Yui, T., & Ogawa, K. (1997). Molecular and crystal structure
16 of hydrated chitosan. *Macromolecules*, 30(19), 5849-5855.
- 17 Pandis, Christos, Madeira, Sara, Matos, Joana, Kyritsis, Apostolos, Mano, João F., & Ribelles, José Luis
18 Gómez. (2014). Chitosan-silica hybrid porous membranes. *Materials Science and*
19 *Engineering: C*, 42(0), 553-561. doi: <http://dx.doi.org/10.1016/j.msec.2014.05.073>
- 20 Pandis, Christos, Spanoudaki, Anna, Kyritsis, Apostolos, Pissis, Polycarpos, Rodriguez Hernandez, Jose
21 Carlos, Gomez Ribelles, Jose Luis, & Monleon Pradas, Manuel. (2011). Water Sorption
22 Characteristics of Poly(2-hydroxyethyl acrylate)/Silica Nanocomposite Hydrogels. *Journal of*
23 *Polymer Science Part B-Polymer Physics*, 49(9), 657-668. doi: 10.1002/polb.22225
- 24 Pradas, M. Monleón, Ribelles, J. L. Gómez, Aroca, A. Serrano, Ferrer, G. Gallego, Antón, J. Suay, &
25 Pissis, P. (2001). Interaction between water and polymer chains in poly(hydroxyethyl
26 acrylate) hydrogels. *Colloid and Polymer Science*, 279(4), 323-330. doi:
27 10.1007/s003960000426
- 28 Ravi Kumar, Majeti N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional*
29 *Polymers*, 46(1), 1-27. doi: [http://dx.doi.org/10.1016/S1381-5148\(00\)00038-9](http://dx.doi.org/10.1016/S1381-5148(00)00038-9)
- 30 Ravindra, R., Krovvidi, Kameswara R., & Khan, A. A. (1998). Solubility parameter of chitin and
31 chitosan. *Carbohydrate Polymers*, 36(2-3), 121-127. doi: [http://dx.doi.org/10.1016/S0144-](http://dx.doi.org/10.1016/S0144-8617(98)00020-4)
32 [8617\(98\)00020-4](http://dx.doi.org/10.1016/S0144-8617(98)00020-4)
- 33 Rinaudo, Marguerite. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer*
34 *Science*, 31(7), 603-632. doi: <http://dx.doi.org/10.1016/j.progpolymsci.2006.06.001>
- 35 Samuels, Robert Joel. (1981). Solid state characterization of the structure of chitosan films. *Journal of*
36 *Polymer Science: Polymer Physics Edition*, 19(7), 1081-1105. doi:
37 10.1002/pol.1981.180190706
- 38 Sashiwa, Hitoshi, & Shigemasa, Yoshihiro. (1999). Chemical modification of chitin and chitosan 2:
39 preparation and water soluble property of N-acylated or N-alkylated partially deacetylated
40 chitins. *Carbohydrate Polymers*, 39(2), 127-138. doi: [http://dx.doi.org/10.1016/S0144-](http://dx.doi.org/10.1016/S0144-8617(98)00167-2)
41 [8617\(98\)00167-2](http://dx.doi.org/10.1016/S0144-8617(98)00167-2)
- 42 Van Krevelen, D. W., & Te Nijenhuis, K. (2009). Chapter 7 - Cohesive Properties and Solubility. In D.
43 W. V. K. by & K. T. Nijenhuis (Eds.), *Properties of Polymers (Fourth Edition)* (pp. 189-227).
44 Amsterdam: Elsevier.
- 45 Viciosa, M. T., Dionísio, M., & Mano, J. F. (2006). Dielectric characterization of neutralized and
46 nonneutralized chitosan upon drying. *Biopolymers*, 81(3), 149-159.

47

48