

Freeze-thaw stability of oil-in-water emulsion stabilized by nanocelluloses

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Abstract. The freeze-thaw stability of emulsions with 40% of vegetable oil and stabilized by three types of nanocelluloses: cellulose nanofibers (CNFs), cellulose nanocrystals (CNCs) and TEMPO cellulose nanofibers (TEMPO-CNFs); were studied by visual observation, optical microscopy and rheological measurements. Cellulose nanocrystals were able to form homogeneous and low viscosity emulsions after freeze and thaw treatment at 0.3% w/v and 0.1 M NaCl. The effect of pH on oiling off was evaluated at values of 4, 5, 6 and 7. Significant differences were observed in oiling off, which represent the amount of free oil present in an emulsion. A better stability between the treatments was obtained at pH 4. Ionic strength effect was evaluated at five salt (sodium chloride) concentrations (0.0 0.01, 0.05, 0.1 and 0.2 M), finding significant differences for all the treatments excepting among 0.1 and 0.2 M, in which there was no oil separation that represent stability. Apparently, the addition of salt enhances the stability of the emulsions at values no more than 0.1 M; above this concentration, a negative effect was observed; this was due to the salting out effect. Cellulose nanocrystals had better emulsifying properties than CNFs and TEMPO-CNFs and can stabilize emulsions after freeze and thaw under specific conditions. It is recommended to study the cellulose nanocrystals behavior in the encapsulation of hydrophobic substances to be added in a hydrophilic matrix after a freeze-thaw treatment.

Key words: Ionic strength, nanocrystals, oiling off, pH.

Resumen. La estabilidad de emulsiones con 40% de aceite vegetal después de un tratamiento de congelación y descongelación y estabilizadas por tres tipos de nanocelulosa: nanofibras de celulosa (CNFs), nanocristales de celulosa (CNCs) y nanofibras de celulosa TEMPO (TEMPO-CNFs); fue estudiada a través de observación visual, microscopía óptica y reología. Los nanocristales de celulosa formaron emulsiones homogéneas y de baja viscosidad después de un tratamiento de congelación y descongelación a una concentración de 0.3% p/v. y a 0.1 M de cloruro de sodio. El efecto del pH en la formación de aceite libre fue evaluado a valores de 4, 5, 6 y 7. Diferencias significativas fueron observadas entre los valores de pH. La mejor estabilidad entre los tratamientos fue obtenida a un pH de 4. El efecto de la fuerza iónica fue evaluado en cinco concentraciones de cloruro de sodio (0.0 0.01, 0.05, 0.1 and 0.2 M), encontrando diferencias estadísticas entre todos los tratamientos excepto entre 0.1 M y 0.2 M, en los que no hubo una separación de aceite lo cual está relacionado con estabilidad. Aparentemente, la adición de sal mejora la estabilidad de las emulsiones a valores no mayores de 0.1 M; arriba de esta concentración un efecto negativo fue observado, esto fue atribuido al efecto “salting out”. Los nanocristales de celulosa tuvieron mejores propiedades emulsificantes que CNFs y TEMPO-CNFs y pudieron estabilizar emulsiones después de un tratamiento de congelación y descongelación bajo condiciones específicas. Se recomienda estudiar el comportamiento de los nanocristales de celulosa en la encapsulación de sustancias hidrofóbicas para ser añadidas en una matriz antes y después de un tratamiento de congelación y descongelación.

Palabras clave: Fuerza iónica, fuga de aceite, nanocristales, pH.

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1. INTRODUCTION

An emulsion can be described as system shaped for two immiscible liquids stabilized by the action of surface-active substances, that are capable to react with the oil-water interface reducing flocculation or coalescence of the droplets, both processes can lead to a layer separation and breakdown of the emulsions structure (Friberg *et al.* 1997; McClements 2005). Many products in the food industry are emulsion-based or contain emulsion. Some belong to oil in water emulsions such as milk, carbonated drinks and soup. Others belong to water in oil emulsion, like margarine and butter (Dalgleish 2006; McClements 2005).

One of the most common preservation technology in food industry is freezing storage, which keeps microorganism in dormant state and extend the shelf life. Though freezing is widely used in long term storage and transport of food, it is detrimental to the stability of emulsions (Friberg *et al.* 1997; McClements 2005). Several physicochemical phenomenon occur when an emulsion system is submitted to a freeze-thaw treatment (FT). Due to nucleation and recrystallization of ice crystals and the high solute concentration, the growing ice modifies the interdroplet forces, leading to the separation of phases (Degner *et al.* 2014; Ghosh and Coupland 2008; Zhao *et al.* 2018). Different factors as lipid composition, sugar concentration, salt concentration or surfactant composition affect the FT stability of emulsions. Thus, with a better designing of emulsion, the quality of some foodstuff can be improved (Degner *et al.* 2014; Ghosh and Coupland 2008).

Amphiphilic biopolymers are able to act as emulsifiers through covering and coating the oil surface in oil-in-water emulsions (Kalashnikova *et al.* 2011; Sun *et al.* 2018). Emulsion stabilized by small particles is defined as Pickering emulsion (Pickering 1907; Ramsden 1903). The particles absorbed at the interface of the emulsions droplets act as a mechanical barrier, giving the ability to disperse high volume fractions of discontinuous phase and enhance the stability of emulsions (Binks 2002; Björkegren *et al.* 2017; Horozov and Binks 2006). If the energy applied into the system is enough to overcome the adsorption energy, Pickering emulsions are able to be created, without reducing the interfacial tension. Nevertheless, particles are also needed to be partially dispersed on both phases (Binks 2002; Björkegren *et al.* 2017).

The most common renewable biopolymer in nature is cellulose and it is present as highly crystalline nanofibrils separated by amorphous regions (Habibi *et al.* 2010). Nanofibrils can be isolated to produce nanocellulose by four ways: mechanical treatments (cellulose nanofibrils CNFs), acid hydrolysis (cellulose nanocrystals CNCs), chemical oxidation (2,2,6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils TEMPO-CNFs) or by bacteria fermentation (*Glucanobacter*) (Gama *et al.* 2013; Klemm *et al.* 2011).

The excellent emulsification properties of nanocellulose and its ability of stabilizing Pickering emulsions, due to its amphiphilic nature, has been recently demonstrated. Specifically this feature is due to the crystal cellulose surface, which originates from the hydrophobic face and hydrophilic edge of cellulose chains (Biermann *et al.* 2001; Glasser *et al.* 2012; Kalashnikova *et al.* 2011; Kalashnikova *et al.* 2012). Due to biocompatibility, biodegradability and low toxicity, nanocellulose is an attractive component in biomedicine and food (Gómez H *et al.* 2016; Hamad 2017; Lin and Dufresne 2014).

The objectives of this study were:

- To determine the FT stability of emulsions prepared by three nanocelluloses (CNFs, CNCs and TEMPO-CNFs).
- To analyze the effect of pH on freeze-thaw stability of oil-in-water emulsions using cellulose nanocrystals.
- To evaluate the effect of ionic strength on freeze-thaw stability of oil-in-water emulsions using nanocellulose.

2. MATERIALS AND METHODS

Materials.

Cellulose nanocrystals (CNFs) (3.0 wt %) were synthesized from natural lignocellulosic fibers by the action of an ultrafine grinder. Cellulose nanofibers (CNCs) (10.4 wt %) were obtained by sulfuric acid hydrolysis, followed by dilution, setting, and concentration with a membrane filtration. TEMPO-CNFs (1.1 wt %) were obtained by the TEMPO catalyzed hypochlorite oxidation at pH 10.0. All three nanocelluloses were manufactured by the USDA Forest Products Laboratory and purchased from the Process Development Center at the University of Maine (Orono, Me, USA). The surface charge density of CNCs was 0.36 mmol $-\text{OSO}_3\text{Na/g}$ dry mass. The surface charge density of TEMPO-CNFs was 1.40 mmol $-\text{COONa/g}$ dry mass. All nanocellulose used were in slurry form. Vegetable oil (soybean) purchased from a local grocery. Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Deionized (D.I) water was used throughout the experiment.

Preparation of oil-in-water emulsions.

Sodium chloride was dissolved into deionized water with concentration varied from 0-0.2 M. Nanocellulose was added into NaCl solution and its final concentration varied from 0.1% to 0.5%. Finally, vegetable oil was added until a final concentration of 40%. The pH values were adjusted to 4.0, 5.0, 6.0 or 7.0 with sodium hydroxide and hydrochloric acid. The mixtures were processed by a homogenizer (Kinematica Polytron PT 10/35 GT-D) at 10,000 rpm for 2 min followed by 2 min sonification at amplitude of 60% in an ice bath (VCX 750, Sonics & Materials, Newton, CT).

Freeze-thaw protocol.

Emulsions stabilized by nanocellulose (15 ml) were transferred to vertical glass containers and submitted to freezing for 24 h at $-20\text{ }^\circ\text{C}$ in a temperature-controlled refrigerator. Then, the samples were thawed at $4\text{ }^\circ\text{C}$ for 24 h in the same refrigerator. An emulsion kept at $4\text{ }^\circ\text{C}$ for 48 h without FT treatment was used as the controls for each treatment.

Physicochemical nanocellulose properties influence in FT o/w emulsions.

The emulsions were made by three nanocelluloses with concentration varied from 0.1-0.5 wt%. The ionic strength was 0.1 M and the content of oil fraction was 40% for all the samples. The pH values were adjusted to 4.0 and one FT cycle was applied. The stability of emulsion was evaluated by visual appearance, oiling off, creaming index, microstructure, rheology and particle size.

Influence of pH on FT o/w emulsions stabilized by CNCs.

The samples were prepared with 0.3% of CNCs. The ionic strength was 0.1 M (NaCl) and content of oil fraction was 40%. The pH was adjusted to 4.0, 5.0, 6.0 and 7. One FT cycle was applied. Stability of emulsions were analyzed by visual appearance, oiling off, creaming index, microstructure, rheology and particle size.

Ionic strength effect on FT o/w emulsions stabilized by cellulose nanocrystals.

The samples were prepared with 0.3% of CNCs with 40% oil fraction. The ionic strength was adjusted to 0, 0.01, 0.05, 0.1 and 0.2 M. The pH values were adjusted to 4.0. One FT cycle was applied. The emulsion stability was evaluated by appearance, oiling off, creaming index, microstructure, rheology and particle size.

Measurement of emulsion properties.

Oiling off.

The free oil were measured based on the method described by (Palanuwech *et al.* 2003) with some modifications using Sudan III (Solvent Red 23). Sudan III was prepared as a stock solution with concentration of 0.0015 wt%. The solution was mixed during 12 h and then 4 g were added to 16 g of the emulsion. The emulsions were vortexed for 30 s, incubated for 60 min and then centrifuged at 15,000 g for 20 min at 4 °C. Dyed free oil over the emulsion (1 ml) was transferred into a cuvette and its absorbance was measured at 508 nm by Evolution 201 UV-visible spectrophotometer (Thermo Fisher Scientific., Waltham, MA, USA). Changes in absorbance were attributed to free oil. The percentage of free oil in the emulsion was calculated based on equation 1.

$$\text{Oiling off (\%)}: \left[M_o \times \frac{A - 1}{M_e \times \phi_e} \right] \times 100 \quad [1]$$

Where;

M_o is the mass (g) of the added dye solution (g), M_e is the mass (g) of the emulsion, ϕ_e is the mass fraction of oil in the emulsion, and $A = A_b/A_a$ is the ratio of the absorbance at 508 nm of the dye solution before (A_b) and after (A_a) mixing with emulsions.

Creaming index.

After FT treatment, some samples suffered a phase separation mainly caused by the free oil. A caliper was used to measure the height of the serum layer (H_s) and the total height of the emulsion (H_t) (Zang *et al.* 2019). The emulsion creaming index (CI%) was calculated by equation 2.

$$\text{CI (\%)}: \left[\frac{H_s}{H_t} \right] \times 100 \quad [2]$$

Optical microscopy.

After FT treatment, the microstructure of emulsion was captured by an optical microscope BX51, Olympus, Tokyo, Japan) with a built-in digital camera (DP 70, Olympus, Tokyo, Japan) under 40X magnification. The emulsions were diluted 50 times with sodium dodecyl sulfate (SDS) with a final concentration of 0.5%.

Rheological behavior.

The rheological analysis was used to compare the mechanical properties of the emulsions. Through an oscillatory plate were performed frequency sweep and steady state flow test, at 25 °C with seven point per decade. The frequency range was from 0.1 to 10 (Hz) and the steady state flow range was from 0.01 to 100 (1/s). The rheological parameters obtained were shear stress, shear rate, apparent viscosity, angular frequency, dynamic modules.

Particle size of the oil droplets.

The size distribution of the droplets was measured by a laser diffraction particle size (LS 13 320 SW, Universal Liquid Module). The samples were diluted with SDS 100 times until a final concentration of 1% to avoid droplets coalescence and flocculence. The particle size was expressed as the mean volumetric size d_{34} .

Experimental design.

A Completely Randomized design was used to study the effect of pH of the 4 treatments (pH 4.0, 5.0, 6.0 and 7.0) in order to analyze its influence in oiling off. Three replicates of each treatment gave rise to 12 experimental units, as shown in table 1.

Table 1. pH effect on freeze-thaw emulsions.

pH	Treatments
4	1
5	2
6	3
7	4

To study the ionic strength, five treatments were performed (0, 0.01, 0.05, 0.1 and 0.2 M of sodium chloride) in order to analyze its influence in creaming index (CI). Three repetitions of each treatment gave rise to 15 experimental units, as shown in table 2.

Table 2. Ionic strength effect on freeze-thaw emulsions.

Molarities (NaCl)	Treatments
0	1
0.01	2
0.05	3
0.1	4
0.2	5

Statistical analysis.

An analysis of variance was performed using a Duncan means separation to determine statistical differences between the treatments. The statistical analysis was performed with “Statistical Analysis System” (SAS®) version 9.4.

3. RESULTS AND DISCUSSION

Physicochemical nanocellulose properties influence in freeze-thaw o/w emulsions.

Several chemical and morphology features of nanocellulose might affect the FT stability of o/w emulsions. As shown in figure 1, CNCs and TEMPO-CNFs had not apparent oiling off and therefore excellent stability after FT treatment under 0.1 M sodium chloride and pH 4. On the other hand, CNFs presented oiling off in all the evaluated concentrations before and after FT. In addition, at concentrations of 0.1-0.2%, a separation with three phases was observed (oil-emulsion-water). Apparent differences in stability and homogeneity were attributed to morphological properties of each analyzed nanocellulose. CNFs (1211) had a higher degree of polymerization (DP) than TEMPO-CNFs (196) and CNCs (134). Z-average hydrodynamic diameter values of nanocelluloses can be order with the same pattern (5099.0, 167.3, and 85.1 nm) (Li *et al.* 2019). An inversely proportional relation between emulsion stability and properties such as DP and particle size in polysaccharides was reported before (Akhtar *et al.* 2002; Dickinson 2009; Winuprasith and Suphantharika 2013). This is mainly because small particles are more able to adhere in the lipid surface and perform as Pickering react. Therefore, CNCs formed apparently more homogenous and stable emulsions (Binks 2002; Ridet *et al.* 2016).

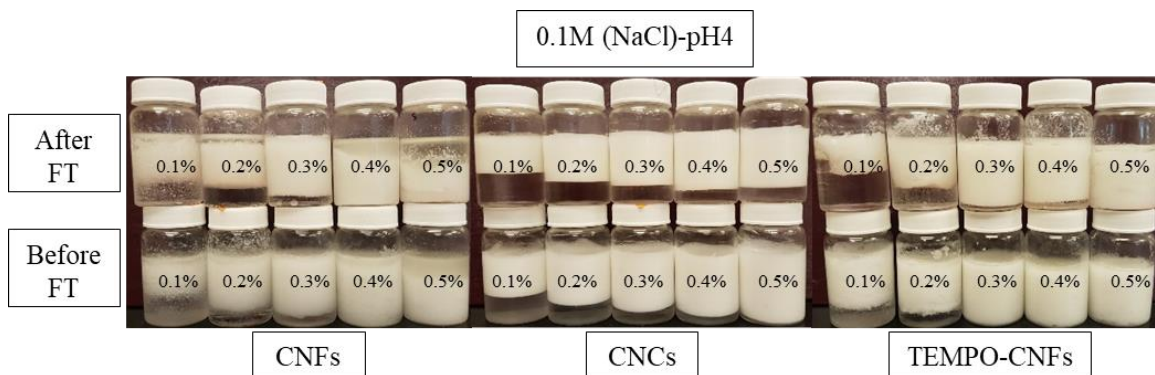


Figure 1. Cellulose nanofibers (CNFs), cellulose nanocrystals (CNCs) and TEMPO cellulose nanofibers (TEMPO-CNFs) freeze-thaw (FT) emulsions at 0.1M (NaCl), pH 4 and several concentrations (0.1, 0.2, 0.3, 0.4, 0.5M).

In the case of CNFs FT samples, a better stability was observed at 0.3% without visual differences among above concentrations. Whereby rheological test and the other phases of the research were made at 0.3% w/v for all the samples. The rheological properties before and after FT treatment of emulsions stabilized by three different nanocelluloses at 0.3% w/v

are shown in Figure 2 and 3. All the emulsions showed a gel-like behavior because the storage modulus (G') were always higher than the loss modulus (G''), and both were slightly dependent of the angular frequency as shown in Figure 2 (Clark *et al.* 1983). This met with previous research about nanocellulose rheology behavior (Zhao *et al.* 2018; Zhao *et al.* 2016). After FT, the modulus values are higher than before FT, whereby, an increased in elasticity and gel strength can be observed for all the samples. Furthermore, there was a higher gel strength and elasticity increasing after FT for CNCs than after FT for TEMPO-CNFs and CNFs. This can be related with the improvement of the FT stability by CNCs and TEMPO-CNFs as demonstrated in Figure 1. All the obtained results agreed with previous research about gel-like mechanism to enhance the FT stability of emulsions (Winuprasith and Suphantharika 2013; Zhao *et al.* 2018; Zhao *et al.* 2016).

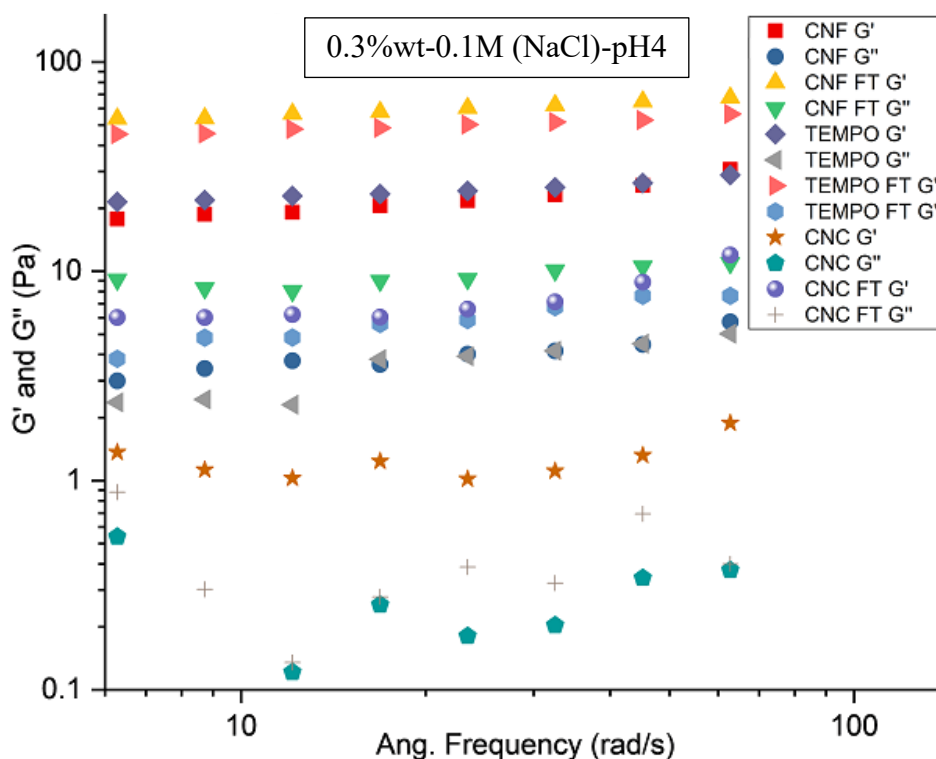


Figure 2. Storage and loss modulus of cellulose nanofibers (CNFs), cellulose nanocrystals (CNCs) and TEMPO cellulose nanofibers (TEMPO-CNFs) before and after freeze-thaw (FT) treatment.

As shown in figure 3, the apparent viscosity of all the samples decreased with an increased shear rate, which is in accordance with the pseudoplastic definition (Hojjat *et al.* 2011). These results were consistent with most polysaccharides viscosity behavior (Lapasin 1995; Zhao *et al.* 2018; Zhao *et al.* 2016). TEMPO-CNFs samples are the most viscous and the only sample with increased apparent viscosity after FT. Samples stabilized by CNCs showed lower viscosity and can be explained by its lowest DP compared with CNF and

TEMPO-CNFs (Harland 1952; Li *et al.* 2019). Biopolymers provided stability by different mechanism, they avoided flocculation and coalesce by inducing repulsive electrostatic interactions or steric hindrance between droplets (Bouyer *et al.* 2012).

Nanocellulose affected the viscosity of the emulsion's continuous phase and this reduced droplets movements and encounters in emulsions (Bouyer *et al.* 2012). CNCs presented the lowest viscosity and was selected for the further experiments.

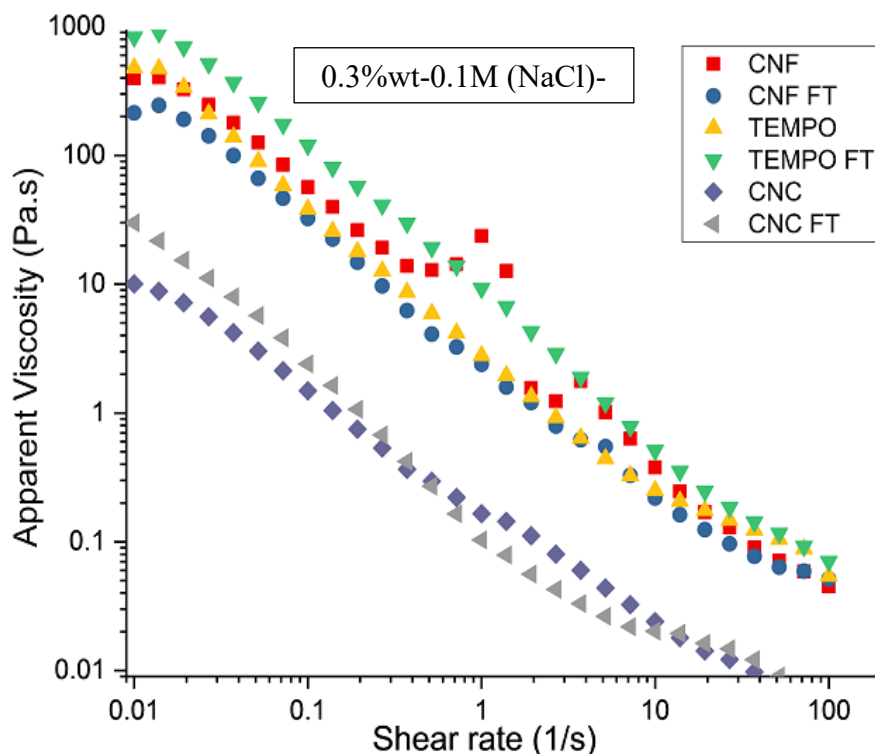


Figure 3. Viscosity of cellulose nanofibers (CNFs), cellulose nanocrystals (CNCs) and TEMPO cellulose nanofibers (TEMPO-CNFs) before and after freeze-thaw (FT) treatment.

Influence of pH on freeze-thaw o/w emulsions stabilized by cellulose nanocrystals.

The emulsion appearance is an important factor that consumers consider when purchasing is carrying out. Also, perception of quality of a product is strongly related with the overall appearance of an emulsion product (Hutchings 1999). All the observed samples showed good homogeneity without oil separation to the surface (Figure 4). However, the aqueous phase separation was evident in all the treatments due to the high oil fraction used (40%). A stability enhancement after FT treatment was observed with decreased pH and with 0.1 M ionic strength. On the other hand, before FT there were not apparent differences among the samples.

Oiling off determines the free fat that is not interacting with the emulsifier and lead the phases separation. High values correspond to an elevated droplets coalescence (Palanuwech *et al.* 2003). It is important to mention that the samples were centrifuged at

15,000 g for 20 min at 4°C with the aim of enhancing the separation of oil from the remainder of the sample.

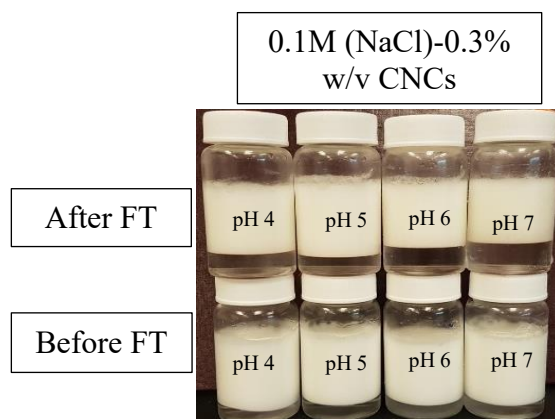


Figure 4. Emulsions stabilized using 0.3% w/v cellulose nanocrystals (CNCs) with 0.1 M (NaCl) at four pH values (4, 5, 6, and 7) after and before freeze-thaw (FT) treatment.

The analysis were performed to FT emulsions stabilized by CNCs at four pHs (4, 5, 6 and 7) with 40% of oil fraction and 0.1 M of ionic strength and the results of the means separation are shown in table 3. There was a statistical difference between all of them. The lowest percentage of oiling off was 5% at pH 4 continued by 31% at pH5, 96% at pH 6, and 77% at pH 7. Three replicates were prepared for each treatment for stadistical purposes.

When oil droplets are under pressure from the ice recrystallization, the interfacial surface becomes the most important resistance to coalescence (Ghosh and Coupland 2008). Surfactant particles are less effective to protect the oil droplet surface compared with polymers which can form thicker layers (Thanasukarn *et al.* 2004). The hypothesis that thicker layers provide better protection to the oil surface is strongly supported by many researches (Aoki *et al.* 2005; Thanasukarn *et al.*2006).

During the freezing process, some cellulose nanocrystals layers were broken and released oil from droplets. However, the strong CNCs adsorption, influenced by the pH of the emulsion, enhanced the stability of FT samples. The nanoparticles adsorption at the emulsion interface surface can decrease with high charge density (Ridel *et al.*2016; Sun *et al.*2018). Prior studies demonstrated that cellulose nanocrystals were negatively charged with 0.4 mmol/g dry mass due to the acid hydrolysis (Kalashnikova *et al.*2012; Li *et al.*2019; Ngwabebhoh *et al.*2018).

Cellulose nanocrystals covered the oil droplets to prevent coalescence and negatively charge the surface (Kalashnikova *et al.*2012). Thus the acidity of the solution determined the amount of protonated particles on the oil droplets surface and while the pH got closer to the pKa (2.46) of CNCs (Wang *et al.* 2011), the number of protonated groups were the same as ionized group, producing a less charge membrane.

Table 3. Oiling off means and standard deviation of freeze-thaw emulsions stabilized by cellulose nanocrystals (0.3% w/v) under pH changes.

Treatments	Oiling Off
	Percentage \pm S.D
pH 4	0.05 ^d \pm 0.00
pH 5	0.31 ^c \pm 0.03
pH 6	0.96 ^a \pm 0.03
pH 7	0.77 ^b \pm 0.03
%C.V.	3.38

^{abc} Different letter in the same column represent statistical difference between the values of each part. Considering $P < 0.05$ and applying a Duncan test.

S.D. Standard Deviation.

C.V. Coefficient of Variation.

Thus, the interdroplet repulsion forces of the surface membrane was reduced due to more acidity without reaching the pKa (Mikulcová *et al.* 2018). Namely, the interface surface was still charged and able to endure the ice crystals growing pressure.

Important properties as appearance, texture, flavor profile, and shelf life of an emulsion are related with oil droplets size. Smaller diameter value of oil droplets represent a better stability in a emulsion system (McClements 2005).

The particle size distribution is reported with the objective of measuring the oil droplets size and its disposition throughout the sample (Figure 5). Samples at pH 4.0 5.0 and 6.0 presented a monomodal distribution which is related with Oswald ripening forces. While pH 7.0 sample showed a bimodal distribution which could be caused by a heterogeneous coalescence. Oswald ripening process is the dissolution smaller particles and the growth of larger particles at the expense of smaller particles (Kabalnov 2001). These results can explain the higher oiling off value of pH 6.0 regarding pH 7.0, as shown in table 3.

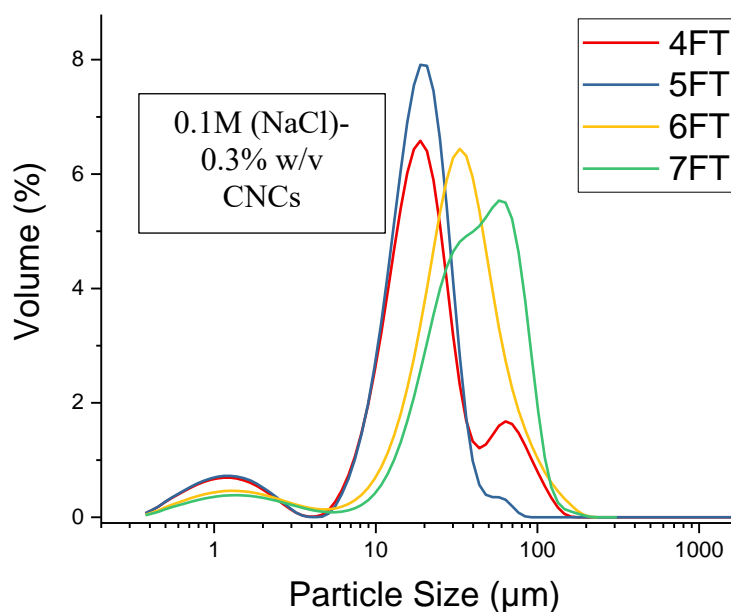


Figure 5. Particle size distribution (PSD expressed as volume frequency) of emulsions stabilized by 0.3% of cellulose nanocrystals (CNCs), at four pH values (4, 5, 6 and 7), 0,1M (NaCl) and 40% of oil fraction after freeze-thaw (FT) treatment.

The Oswald ripening was significant up to pH 6.0 and then, at some point, between pH 6.0 and pH 7.0, the natural coalescence starts to dominate. The particle size distributions are shown in table 4. Droplets diameters above 10 μm can be considered as large oil droplets (McClements 2005) and all the samples met this definition. Sample at pH 4 had the smaller diameter peak, which represent the best stability between the FT samples. The results agreed with the oiling off test. An inversely proportional relation among pH and particle size can be deducted.

Table 4. Peaks and frequency of (PSD) presented in Figure 5.

Curves	Peak (μm)	Volume Frequency (%)
pH 4	4.42	5.98
pH 4 FT	12.99	6.58
pH 5	6.16	6.43
pH 5 FT	18.9	17.91
pH 6	9.82	4.54
pH 6 FT	33.01	6.44
pH 7	10.78	6.36
pH 7 FT	57.77	5.54

PSD. Particle Size Distribution.

FT. After Freeze-Thaw Treatment.

A bias in particle size analysis caused by aggregation of cellulose nanocrystals can be discarded from the optical microscope pictures showed in figure 6. Therefore, the particle size values were attributed to the oil droplets of the emulsion, but not to the aggregation of CNC.

Table 4 shows a significant increase of oil droplets diameter after FT for all pH values, which is consistent with the images shown in figure 6. It can be observed the smallest particle size after FT was observed at pH of 4.0 (being close to pH 7 before freeze-thaw treatment).

Based on all of the performed analysis, it can be concluded that there was a strong relation between pH and the stability of the emulsions studied after FT, improving their behavior in values close to the pKa and the best stability was observed at pH of 4.0.

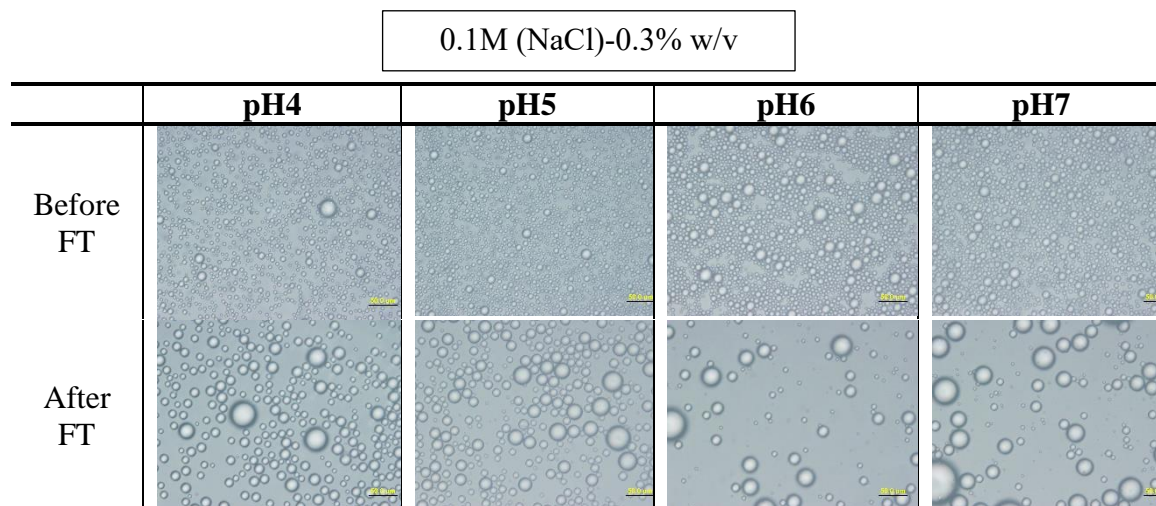


Figure 6. Optical microscope pictures at 40X of emulsion stabilized by cellulose nanocrystals (CNCs) at four pH values after and before freeze-thaw treatment (FT).

Ionic strength effect in FT o/w emulsions stabilized by cellulose nanocrystals.

Many emulsion products use salt in their formulation for several reasons and with different concentrations. Salt concentration is one of the most important factor that affect the stability of an emulsion. Especially because ionic strength modify the interdroplet forces and repulsion between the emulsifier molecules (McClements 2005).

The samples were prepared using a concentration of 0.3% w/v of cellulose nanocrystals at pH4. Several molarities of sodium chloride (0, 0.01, 0.05, 0.1, and 0.2 M) were evaluated before and after freeze-thaw treatment and were presented in figure 7. An important enhancement of CNC emulsifying properties with a higher ionic strength can be observed, even before FT, the sample without NaCl showed an entire phase separation, suppressing the amphipathic behavior of cellulose nanocrystals. It is important to mention that nanocellulose were not able to form an emulsion phase without NaCl.

In solutions at 0.01 M, CNC is in both phases (aqueous and emulsion), while in all the rest of the evaluated concentrations it is only in the emulsion phase leaving below a transparent aqueous phase. However, at concentrations above 0.1 M NaCl, a homogeneous (stable white color in whole emulsion phase) can be obtained after FT, and there were not apparent differences between 0.1 M and 0.2 M.

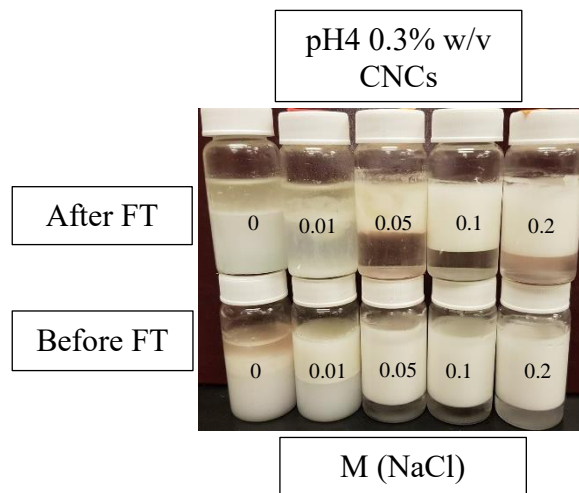


Figure 7. Emulsions stabilized using 0.3% w/v. cellulose nanocrystals (CNCs), pH4 at several ionic strength (0-0.01-0.05-0.1-0.2M).

Several research about modulation of cellulose nanocrystals surface proved that a stable oil-in-water emulsion was not possible if the charge density was more than 0.03 charge/nm^2 , regardless of the source (Capron and Cathala 2013; Cherhal *et al.* 2016; Kalashnikova *et al.* 2012). All of the oil droplets are covered by nanocellulose and therefore they have the same electrical charge. Aggregation can be prevented if the charge is enough to keep the electrostatic repulsion between them. Salt screened the hydrophilic groups of nanocellulose, made it more hydrophobic and able to adhere into the oil membrane (Aoki *et al.* 2005; Carneiro-Da-Cunha *et al.* 2011; McClements 2005; Ridel *et al.* 2016).

Ionic strength also affects the effective size of a biopolymer and their molecular conformation. Many researches have reported an inversely proportional relation between ionic strength and Z-average for all the polysaccharides, excepting alginate (Carneiro-Da-Cunha *et al.* 2011; Wang *et al.* 2011). The importance of the polysaccharide molecular size to Pickering emulsion has been demonstrated earlier on the first phase of this research. Small cellulose nanocrystals with a screen surface charge density are able to adhere onto the oil droplets surface with higher surface coverage and perform as resistant membranes that are capable to endure higher external pressure (Capron and Cathala 2013).

Creaming index (CI) was used to analyze the emulsion stability after the FT treatment. Creaming represent gravitational separation and it was caused by the upward movement of oil droplets due its lower density than the rest of the emulsion (McClements 2005). Some oil droplets did not move upward because a strong interaction between surface of droplets and cellulose nanocrystals existed which kept the droplets into the emulsion phase.

It is important to mention that variations in ionic strength unlike pH, caused oil separation. CI is a relation between the layers height of an emulsion. The values of this analysis are available in table 5. Without salt addition, 40% CI was observed which represent complete instability because this value is the same quantity of oil incorporated to each sample. Hence, without the addition of sodium chloride, cellulose nanocrystals were not able to perform as

a Pickering emulsifier despite of the ultrasound and dispersing process applied. The second highest mean was 25% corresponding to a 0.01 NaCl molarity, followed by 12% using 0.05 M NaCl. Above 0.05 M NaCl, all the oil is absorbed by nanocellulose, avoiding layer separation, whereby the CI means were 0%. All the treatments had significant difference except 0.1 M and 0.2 M, which were statistically similar. These results are consistent with several research (Habibi *et al.* 2010; Kalashnikova *et al.* 2012; McClements 2005) that confirmed the positive effect of an increasing ionic strength in the stability of nanocellulose as emulsifier. For this reason, ionic strength is one of the most important factors, which must be taken into count when an emulsion is subjected to a FT treatment.

Table 5. Creaming index means and standard deviation of freeze-thaw emulsions stabilized by cellulose nanocrystals (0.3% w/v) at pH4 using several molarities of sodium chloride

Treatments	Creaming Index
	Percentage \pm S.D
0M	0.40 ^a \pm 0.05
0.01M	0.25 ^b \pm 0.03
0.05M	0.12 ^c \pm 0.00
0.1M	0.00 ^d \pm 0.00
0.2M	0.00 ^d \pm 0.00
%C.V.	16.21

^{a-d} Different letter in the same column represent statistical difference between the means of each part, considering $P < 0.05$ and applying a Duncan test.

S.D. Standard Deviation.

C.V Coefficient of Variation.

Oiling off test was performed to compare the stability of emulsions after and before freeze-thaw treatment under several molarities, the results are shown in figure 7. An evident salt in and salt out effect can be appreciated in the emulsify properties of FT emulsions. Salt decreased the amount of free oil in the emulsion, until a minimum point at 0.1 M, from which the oiling off value start to increase, therefore the addition had a negative effect at values greater than 0.1 M. This phenome have been observed before in several polysaccharides used as emulsifiers including nanocellulose (Carneiro-Da-Cunha *et al.* 2011; Phan-Xuan *et al.* 2016; Wang *et al.* 2011; Xu *et al.* 2017).

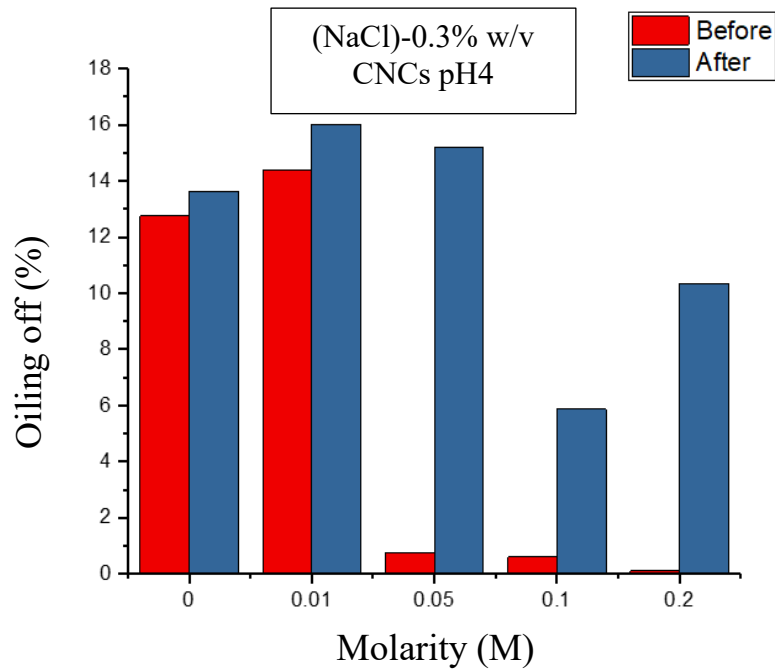


Figure 8. Oiling Off index of emulsions stabilized by cellulose nanocrystals (CNCs) after and before freeze-thaw (FT) at several molarities (0, 0.01, 0.05, 0.1, 0.2 M) of sodium chloride

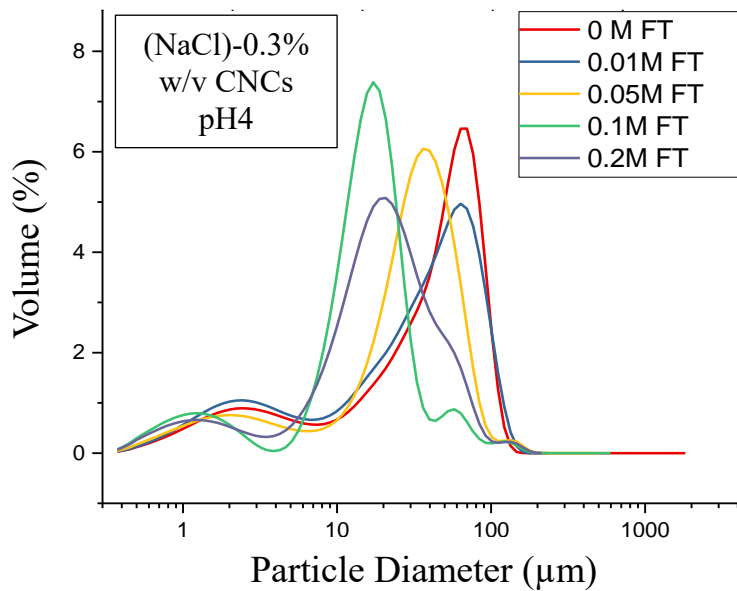


Figure 9. Particle size distribution (expressed as volume frequency) of freeze-thaw (FT) emulsions stabilized by 0.3% of cellulose nanocrystals (CNCs), at five molarities (0, 0.01, 0.05, 0.1 and 0.2M) of sodium chloride, pH4 and 40% of oil fraction.

Figure 9 shows the particle size distribution (PSD, expressed as volume frequency) of FT emulsions stabilized by 0.3% of CNC at five molarities (0, 0.01, 0.05, 0.1 and 0.2 M) at pH 4 and 40% of oil fraction. Table 6 shows the peaks and frequency of each distribution curve. All the curves presented monoidal distribution which is related with Oswald ripening forces acting into the emulsion system (McClements 2005).

The largest particle size after FT was observed at 0 M NaCl. However, the results suggest that the larger particle size of this treatment must not be attributed to damage caused by FT, but to the inability of CNC to cover the surface oil droplets, since the peak of the sample before FT is apparently the same as the one after. The smallest particle size was obtained at 0.1 M NaCl, this met with the oiling off test and indicated that concentrations above this value could damage the emulsion stability, resulting in large oil droplets. These results are in accordance with other studies (Capron and Cathala 2013; Cherhal *et al.* 2016). A direct relation between particle size of the droplets and ionic strength can be concluded before and after FT.

Table 6. Peaks and frequency of PSD presented in Figure 9.

Curves	Peak (μm)	Volume Frequency (%)
0M	69.62	5.93
0M FT	69.62	6.46
0.01M	76.43	7.74
0.01M FT	63.42	4.97
0.05M	9.82	5.64
0.05M FT	36.24	6.06
0.1M	6.76	6.52
0.1M FT	17.18	7.38
0.2M	4.66	6.62
0.2M FT	20.71	5.08

PSD. Particle Size Distribution.

FT. After Freeze-Thaw treatment.

M. Molarity

A bias can be discarded in particle size analysis due to aggrupation of cellulose nanocrystals by the optical microscope pictures shown in figure 10. Therefore, the particle measure can be attributed to the oil droplets. Besides, in the microstructure pictures, the largest droplets were observed at 0 M and there was an increase of oil droplets concentration to more molarity. The microstructure pictures met with creaming index, oiling off and particle size.

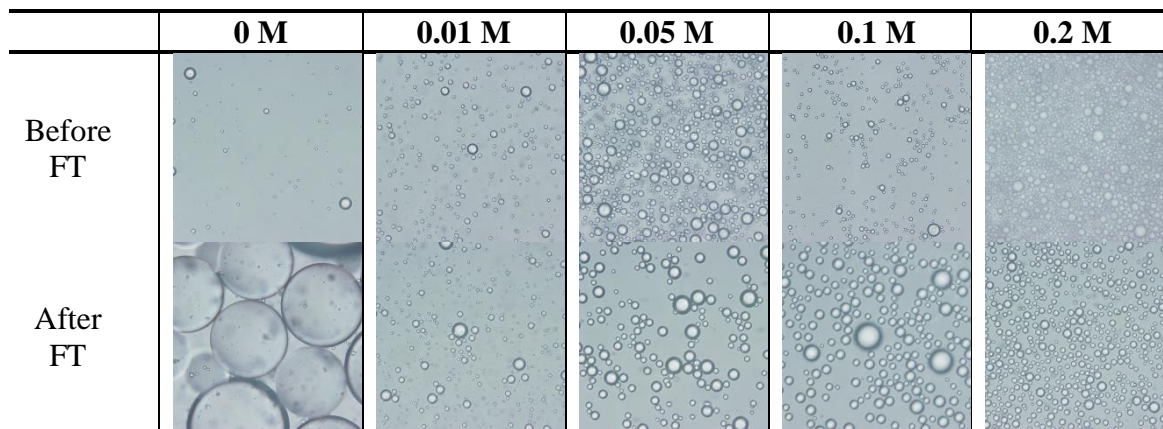


Figure 10. Optical microscope pictures at 40x of freeze-thaw (FT) emulsion stabilized by cellulose nanocrystals at four pH values.

4. CONCLUSIONS

- Cellulose Nanocrystals were effective to form apparently homogeneous emulsions without large visual differences between 0.3, 0.4 and 0.5% w/v
- Oiling off was affected by pH; cellulose nanocrystals presented better stability at acid pH values.
- Addition of salt improve the freeze-thaw stability of emulsions when cellulose nanocrystals perform as surfactant agent.
- Salt concentration above 0.1 M NaCl affect the stability of the emulsions when cellulose nanocrystals perform as surfactant agent and this could be due to salting out effect.

5. RECOMMENDATIONS

- Study the free-thaw stability of oil-in-water emulsions using completely hydrophobic substances due oil vegetable contains some emulsifier components as proteins.
- Study the nanocellulose behavior in the encapsulation of hydrophobic substances to be added in a hydrophilic matrix.

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