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EFFECT OF pH AND CONCENTRATION OF MILK FAT GLOBULE MEMBRANE ON THE RELEASE OF NaCl FROM $W_1/O/W_2$ MULTIPLE EMULSION

EFECTO DEL pH Y LA CONCENTRACIÓN DE MEMBRANA DE GLÓBULO DE GRASA LÁCTEA SOBRE LA LIBERACIÓN DE NaCl EN EMULSIONES MÚLTIPLES $W_1/O/W_2$

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Abstract

This work aimed to investigate the effect of pH and milk fat globule membrane concentration on the release of NaCl from $W_1/O/W_2$ multiple emulsions. Multiple emulsions (ME) were formulated containing carboxymethylcellulose (CMC) and NaCl in the internal aqueous phase. Multiple emulsions were formulated with water and canola oil, as well as polyglycerol polyricinoleate (PGPR) and milk fat globule membrane (MFGM) as emulsifiers for the primary emulsion (W_1/O) and the multiple emulsion ($W_1/O/W_2$) respectively. All MEs containing NaCl showed stability during storage time (28 days). The size ($d_{3,2}$) of the ME was affected by the concentration of MFGM in the external aqueous phase as well as the pH. Emulsions formulated with higher concentrations of MFGM (5%, 6%, and 6.4%) had greater stability. The release of NaCl was observed to decrease at concentrations of 6% MFGM, and pH 5.0, 6.0 and 7.0. ME formulated at different concentrations of MFGM and different pH levels, exhibited increases in modulus G' and G'' as a function of the deformation, showing values of G' higher than those of G'' with up to 10% deformation.

Keywords: emulsion, encapsulation, rheology, release, milk fat globule membrane.

Resumen

Este trabajo tuvo como objetivo investigar el efecto del pH y la concentración de membrana de glóbulo de grasa láctea sobre la liberación de NaCl en emulsiones múltiples $W_1/O/W_2$. Se formularon emulsiones múltiples (EM) conteniendo CMC y NaCl en la fase acuosa interna. Las emulsiones múltiples fueron formuladas con agua y aceite de canola, así como también polirricinoleato de poliglicerol (PRPG) y membrana del glóbulo de grasa láctea (MGGL) como emulsificantes para la emulsión primaria (W_1/O) y para la emulsión múltiple ($W_1/O/W_2$) respectivamente. Todas las EMs conteniendo NaCl presentaron estabilidad durante el tiempo de almacenamiento (28 días). El tamaño ($d_{3,2}$) de las EM se vio afectado por la concentración de MGGL en la fase acuosa externa así como también por el pH. Las emulsiones formuladas con altas concentraciones de MGGL (5%, 6% y 6.4%) presentaron la mayor estabilidad. Con respecto a la liberación de NaCl se observó que a concentraciones de 6% de MGGL y a pH 5.0, 6.0 y 7.0 ésta disminuyó. Las EM formuladas a diferentes concentraciones de MGGL y pH, exhibieron incrementos en los módulos de G' y G'' en función de la deformación, presentando valores de G' mayores a aquellos de G'' hasta un 10% de deformación.

Palabras clave: emulsión, encapsulación, reología, liberación, membrana del glóbulo de grasa láctea.

1 Introduction

Multiple emulsions (ME) are complex liquid multiphase systems. These complex structures allow their use in different applications, such as: separation processes (Gaitzsch *et al*., 2011), encapsulation of carotenoids (Rodriguez-Huezo *et al*., 2004), ω-3 oils (Onuki *et al*., 2003; Cournarie *et al*., 2004), bioactive hydrophilic components, such as

vitamin B (Fechner *et al.*, 2007; Kukizaki y Goto, 2007), immunoglobulins (Lee *et al.*, 2004), insulin (Cournarie *et al.*, 2004), proteins (Su *et al.*, 2006), and amino acids (Weiss *et al.*, 2005). In multiple emulsions, due to the presence of two aqueous domains separated by a layer of oil, the internal aqueous compartment offers great potential for the encapsulation and controlled hydrophilic bioactive ingredient release (Sapei *et al.*, 2012.). Although these have many advantages, its production is limited in practice (Lutz *et al.*, 2009). One of the major disadvantages is its relatively low stability, as it usually contains a high amount of large oil droplets, besides being very sensitive to changes in pH and temperature (Fechner *et al.*, 2007; Sapei *et al.*, 2012). The encapsulation and release of NaCl in multiple emulsions have presented good results; however, these properties are only evaluated at pH values close or equal to 7.0 (Reitberg *et al.*, 2012, Sapei *et al.*, 2012; Lutz *et al.*, 2009). Achieving the encapsulation of hydrophilic bioactive agents in multiple emulsions at different pH ranges is complicated because as mentioned above, these are sensitive to pH changes. Recent studies have reported that the progressive increase of pH in the range of 3-9 improved retention of diacetyl in emulsions made with egg yolk powder and sodium octenyl succinate starch. While in flavored samples α-pinene, higher and lower retention times and low pH 9 and 7 were detected (Bortnowska, 2012). To address the problem of low stability of multiple emulsions (ME) and to incorporate them into food systems in recent years, ME have been developed in which the use of two or more biopolymer combinations protein/polysaccharide are included (Hernández-Marín *et al.*, 2013), materials recovered from by-products of the dairy industry, such as milk fat globule membrane (MFGM) (Dzul-Cauich *et al.*, 2013), among others, in order to reduce the interfacial tension between the oil - aqueous phase of the ME and make these systems more stable. Due to its original function of stabilizing the fat globule in milk, MFGM is regarded as a material with excellent emulsifying capacity. It is concluded that there may be a strong interaction between the surface and the phospholipids that compose the MFGM which could be involved in reducing the interfacial tension.

Due to its high encapsulation efficiency, the potential for the use of ME in food matrices appears promising. However, storage stability at different pH conditions should be determined before its successful use in food. Currently, there are no reports on the release of NaCl from multiple emulsions formulated with MFGM at different concentrations and under different pH conditions. In relation to the above, the objective was to evaluate the concentration of milk fat globule membrane and the effect of pH on the stability and release of NaCl from multiple emulsions.

2 Materials and methods

2.1 Materials

Canola oil (AC; Capullo®, Unilever de Mexico, S.A. de C.V.); Carboxymethylcellulose (CMC; CMC® CEROL 50 000; Grupo Dermet, S.A. de C.V.); lipophilic emulsifier (PGPR; Grinsted® PGPR 90, polyglycerol fatty acid esters and polyricinoleate; Danisco Mexico, S.A. de C.V.); hydrochloric acid, sodium hydroxide (JT Baker, SA de CV) were bought in Mexico City, Mexico. Deionized distilled water was used for all experiments.

2.2 Preparation of multiple emulsions containing NaCl

Multiple emulsions were prepared at 20 ± 2 °C using a two-stage emulsification process (Lobato-Calleros *et al.*, 2009). In the first stage a primary emulsion W_1/O type containing 30 g of dispersed aqueous phase W_1 (25.67 g of deionized water + 0.7 g of CMC + 3.63 g NaCl) and 70 g of the continuous oil phase (O was prepared) (66 g canola oil + 4.0 g of lipophilic emulsifier). The required amounts of CMC and NaCl were hydrated in water for 30 min, at room temperature (20 ± 2ºC). Separately, the EL was dispersed in oil (AC) at room temperature. Subsequently, the internal aqueous phase W_1 was added dropwise to the O oil phase using a homogenizer (Ultra-turrax® T50 basic IKA Works, Inc., Wilmington) operated at 6400 rpm for 5 min. In the second stage, the W_1/O (30 g per 100 g) primary emulsion was re-emulsified at 5800 rpm for 4.0 minutes in MFGM (obtained from fresh raw cream in previous work) dispersions corresponding to different pH levels. During the emulsification process, an ice bath was used to prevent the temperature rise above 20 ± 2 °C.

2.3 Morphology and droplet size of emulsions

The morphology of the emulsion droplets was determined using the light microscope (Olympus BX45, Olympus Optical Co., Tokyo, Japan) adapted with an image analyzer (Olympus digital camera C3030, Olympus America Inc.) system. The average surface volume diameter ($d_{3,2}$) of the droplets of emulsions and its evolution with storage time (0, 7, 14, 21 and 28 days) was determined with a particle size analyzer (2600 Series, Malvern Instruments, Malvern, Worcestershire, UK).

2.4 Release properties

The release of the salt of multiple emulsions $W_1/O/W_2$ was measured using a conductivity model (HI 9835 Hanna Instruments, Woonsocket, USA). Before carrying out release measurements of NaCl, the equipment was calibrated with a standard saline solution of 3.3% NaCl (seawater) (HI 7037 Hanna Instruments, Woonsocket, USA). To carry out the measurements, the electronic conductivity meter was added to a sample of 50 mL of each emulsion. The readings obtained with the equipment as a function of time became the concentration of NaCl, using the concentration NaCl of seawater (3.3% NaCl) as a reference. The NaCl fraction released was calculated according to the following equation (Bonnet *et al.*, 2009):

$$FL(\%) = (C_t \times \phi_e / C_0 \times \phi_i) \times 100 \qquad (1)$$

Where
$FL(\%)$ is the percentage of sodium chloride released from the internal water droplets to the external aqueous phase, C_t is NaCl concentration measured in the external aqueous phase at time t release, C_0 is the initial concentration of NaCl encapsulated within the internal droplets, ϕ_e and ϕ_i are the mass fraction of the external aqueous phase and the internal water droplets in the emulsion, respectively.

2.5 Rheological properties of emulsions

The rheological behavior of the emulsions was determined in freshly prepared emulsions and 14 days of storage with Physica MCR 300 Rheometer using a cone-plate geometry, in which the rotating cone was 50 mm diameter and an angle of 1°. Samples of the emulsions were placed in the rheometer and allowed to stand for 15 minutes to recover structure and for

temperature equilibration (25 °C). Amplitud sweep (0.01-100% strain) at a constant frequency of 1 Hz was performed to determine the linear viscoelastic region, where the rheological properties are not dependent on strain or deformation (Hernández-Rodríguez *et al.*, 2017). The storage or elastic modulus (G') the loss or viscous modulus (G") and the Tan (delta) or loss tangent (G"/G') were evaluated using the software from the equipment (Ortiz -Zarama *et al.*, 2016).

2.6 Experimental design for the response surface method

To analyze the effect of pH and concentration of the milkfat globule membrane (MFGM) on the characteristics of multiple emulsions (size, percent release of NaCl), we used a response surface methodology using a central composite design with factorial design 2^2 with central and axial points. The values of the independent variables (pH and MFGM) varied from pH 5 to 7 and from 4% to 6% MFGM. All experiments were performed in duplicate. The response surface design was applied to the experimental data using the statistical package Statgraphics Plus software (Statistical Graphics Corp., Manugistics, Inc., Cambridge, MA, USA).

3 Results

3.1 Morphology and droplet size of emulsions

The morphology of the droplets of different emulsions did not vary with storage time. This consisted of numerous water droplets inside the oil droplets, which are dispersed in a continuous aqueous phase, which according to Garti (1997) corresponds to type C (Fig. 1).

Multiple emulsions (ME) formulated at low concentrations of MFGM exhibited larger droplet sizes ($d_{3,2}$) than those made with the highest percentage of MFGM. The droplet size ($d_{3,2}$) of the emulsions showed a slight increase at day 1 and remained stable until day 21. At 28 days, the emulsions: $ME_{pH\ 5.0\ MFGM\ 4.0}$, $ME_{pH\ 7.0\ MFGM\ 4.0}$, $ME_{pH\ 6.0\ MFGM\ 5.0}$, $ME_{pH\ 6.0\ MFGM\ 3.6}$ $ME_{pH6.\ 0\ MFGM\ 6.4}$ showed small increases and some emulsions ($ME_{pH\ 7.0\ MFGM\ 6.0}$, $ME_{pH\ 7.4\ MFGM\ 5.0}$ and $ME_{pH\ 4.6\ MFGM\ 5.0}$, showed a slight decrease (Fig. 2). These results suggest that at least all ME were

stable over the storage time. Emulsions formulated with higher concentrations of MFGM (5%, 6%, and 6.4%) had greater stability because the droplet size varied slightly during the storage time. Dzul-Cauich *et al*. (2013) reported that the concentration of MFGM in the external aqueous phase influences the stability
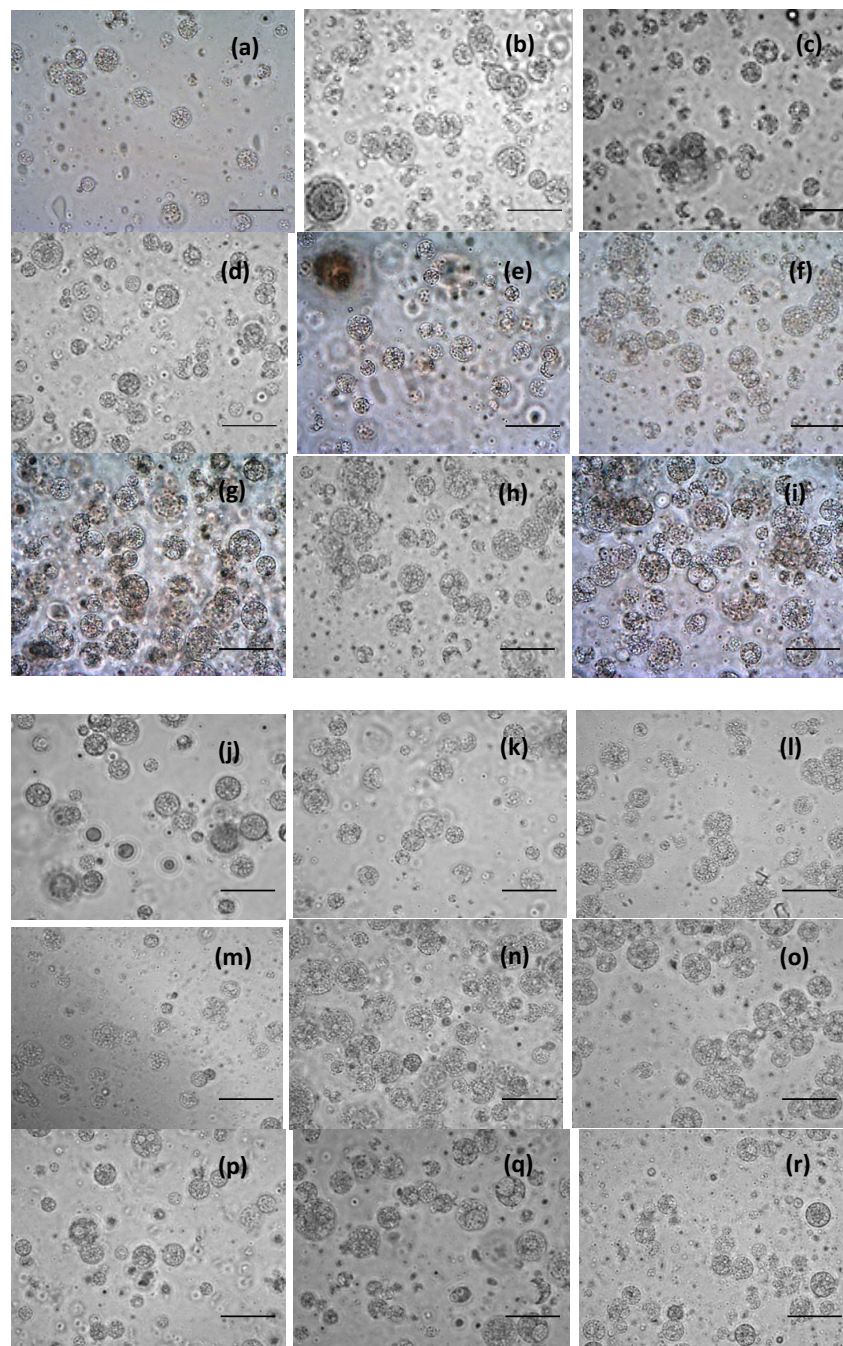

Fig. 1. Optical microscopy images of drops of ME, constituted by spherical oil droplets containing a large number of water droplets after 1 day (a-i) and 28 days (j-r): (a) y (j): ME $_{pH\ 5.0\ MF\ 4.0}$; (b) and (k): ME$_{pH\ 5.0\ MF\ 6.0}$; (c) and (l): ME$_{pH\ 7.0\ MF\ 4.0}$; (d) and (m): ME$_{pH\ 7.0\ MF\ 6.0}$; (e) and (n): ME$_{pH\ 6.0\ MF\ 5.0}$; (f) y (o): ME$_{pH\ 7.4\ MF\ 5.0}$; (g) and (p): ME$_{pH\ 4.6\ MF\ 5.0}$; (h) and (q): ME$_{pH\ 6.0\ MF\ 3.6}$; (i) and (r): ME$_{pH\ 6.0\ MF\ 6.4}$; Scale bar = 10 μm.
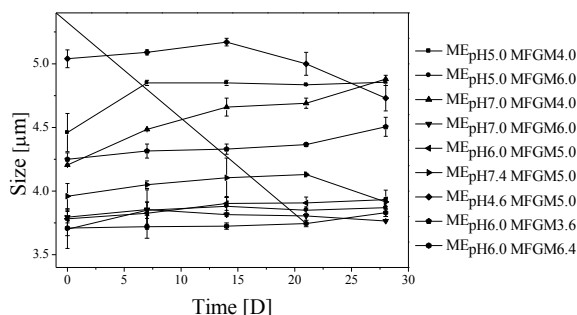
Fig. 2. Change in the droplet size of the ME with respect to storage time.
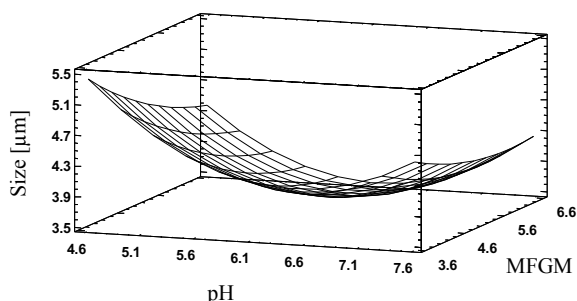

Fig. 3. Response surface graph for the effect of pH and concentration MFGM on the size of the ME.

of the ME and the values of $d_{3,2}$ and with lower concentrations, larger ME drops are obtained. In Figure 1, we observe the ME micrographs taken after 1 day of preparation and 28 days' storage, showing the behavior of the droplet size of the different ME observed.

Figure 3 shows the effects of pH and concentration of MFGM on droplet size of the ME. These two factors had an effect on the droplet size, the pH had a greater effect. Regarding MFGM, we observed that at high concentrations (5%, 6%, and 6.4%) droplet size decreases, which can be explained because there is a greater amount of MFGM to cover the droplets. Dzul-Cauich *et al.* (2013) reported that ME formulated with 6% MFGM yielded smaller droplets, compared to those made with 4% and 5%. Other authors have reported that the concentration of emulsifier is an important factor to achieve droplet sizes with lower diameters, when the concentration of surfactant is increased, the droplet size tends to be smaller due to the reduction of interfacial tension (Tadros *et al.*, 2004; McClements, 2005). Furthermore, it was observed that the pH presented an inversely proportional droplet size effect, i.e., when the pH increases, the droplet size decreases, which can be explained, in that as

MFGM moves away from its isoelectric point (pH = 4.8), this presents greater adsorption capacity at the oil-water. According to López *et al.* (2017), the zeta potential of the MFGM-rich ingredient was negative for pH higher than pI and positive for pH lower than pI .Microstructural analyses revealed the formation of complexes between lipids and proteins for pH around the isoelectric point of the ingredient. Corredig and Dalgleish (1998) found that when the pH decreases, the average droplet size in primary oil-in-water formulated with MFGM increases. According to Petursson *et al.* (2004), proteins tend to stabilize the drops by combining steric and electrostatic repulsions, its efficacy being particularly sensitive to pH.

The regression equation of droplet size as a function of pH and concentration of MGGL (effects with *p* value < 0.05) is presented as follows:

$$Size\,(\mu m) = 23.2628 - 4.91213 pH - 1.37004 MFGM$$
$$+ 0.357871 pH^2 + 0.075 pHxMFGM$$
$$+ 0.0670546 MFGM^2 \qquad (2)$$

The determination coefficient R^2 was 0.83 with *p* <0.05.

3.2 NaCl release

In Figure 4, different ME and their behavior with respect to the NaCl fraction released during the storage period analyzed (1-28 d) may be observed. In general, an increased release of NaCl was observed. The order of behavior of the released fraction of NaCl from the different ME under conditions of pH and percent concentration of MFGM is presented as follows: $ME_{pH\,7.0\,MFGM\,6.0}$, < $ME_{pH\,5.0\,MFGM\,6.0}$, < $ME_{pH\,6.0\,MFGM\,6.4}$, < $ME_{pH\,7.0\,MFGM\,4.0}$, < $ME_{pH\,6.0\,MFGM\,5.0}$, < $ME_{pH\,5.0\,MFGM\,4.0}$, < $ME_{pH\,7.4\,MFGM\,5.0}$, < $ME_{pH\,6.0\,MFGM\,3.6}$, <$ME_{pH\,4.6\,MFGM\,5.0}$.

The pH and concentration of the MFGM of the ME showed different effects on the release of NaCl. The pH presented an inversely proportional linear effect, while the concentration of MFGM was directly proportional to NaCl. In other words, when the MFGM increases, the percent release of NaCl also increases. The opposite case occurred with pH, since by increasing pH the NaCl fraction released decreased, thus indicating that the pH was the factor with the greatest effect on the release of NaCl from ME. However, it was observed that 6% of MFGM and pH 5.0, 6.0, and 7.0 of the NaCl fraction released decreased.
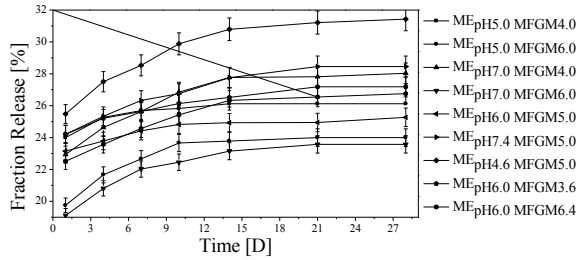
Fig. 4. The behavior of the NaCl fraction released during the storage time of the different ME formulated with MFGM.
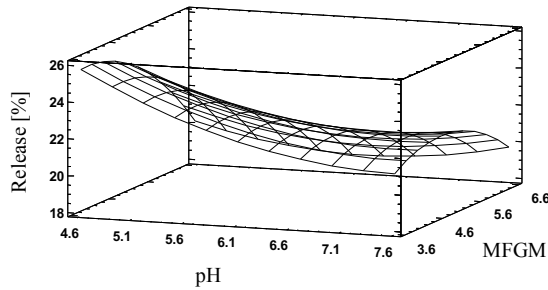

Fig. 5. Response surface graph for the effect of pH and MFGM concentration on the released fraction of NaCl from the ME.

The above could be explained according to Sakurai *et al*., (2009) where the authors mention that the proteins found at pH below its isoelectric point in a state that could be called "closed" while at pH 6.0 there is a high proportion of proteins found in an "open" state. Furthermore, at pH 7.0 more hydrophobic groups are exposed, which may allow the adsorption of the proteins present in the MFGM at the oil-water interface. The above may be because the ME that were elaborated at a higher pH than the isoelectric point of MFGM proteins had a lower release of NaCl, a higher concentration of MFGM adsorbed at the oil-water interface functions as a barrier that creates mass transfer resistance, as reported by Lubbers *et al*., (1998).

The regression equation of the behavior of the released NaCl fraction from ME as a function of pH and concentration of MGGL (effects with p value <0.05) is presented as follows:

$$Release\,(\%) = 48.2258 - 9.17334pH + 2.88468MFGM$$
$$+ 0.484921pH^2 + 0.4725pHxMFGM$$
$$- 0.677069MFGM^2 \qquad (3)$$

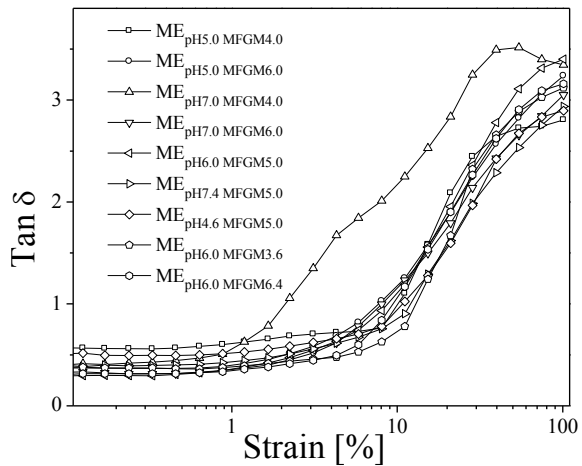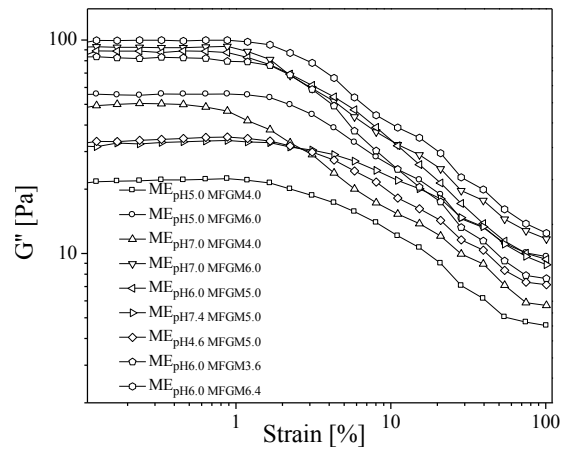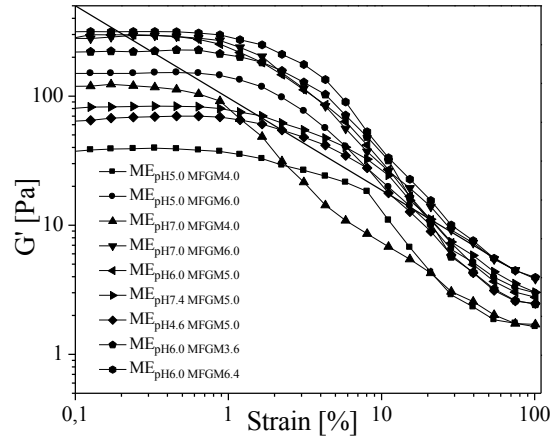The determination coefficient R^2 was 0.68 with $p < 0.05$

Fig. 6. Strain dependence of G', G'' and $\tan\delta$ of ME.

3.3 Rheological properties of emulsions

Dynamic oscillatory shear testing of small amplitude is a useful tool that provides information about the microscopic structure of viscoelastic materials such

as emulsions. This type of testing obtains the values of storage modulus (G'), and loss modulus (G'') of the system. G' is proportional to the magnitude of the elastic component, and G'' is proportional to the magnitude of the viscous component (Wulff-Pérez *et al.*, 2011). ME formulated at different concentrations MFGM and different pH, exhibited increases in modulus G' and G'' as a function of the deformation, showing values of G' higher than those of G'' to 10% strain (Fig. 6). Both G' and G'' were dependent on the deformation. The type of behavior shown in Figure 6 is typical of concentrated emulsions that have a weak gel structure, which arises from flocs held together strongly by polymer bridges but is susceptible to rearrangement during storage and in the presence of shear fields (Dickinson and Pawlowsky, 1996; Barnes, 2004). Based on the G' values of different emulsions, the maximum deformation which took place in the linear viscoelastic region ranged as follows: $ME_{pH\ 5.0\ MF\ 3.6}$ (0.6%) = $ME_{pH\ 5.0\ MF\ 6.0}$ (0.6%) > $ME_{pH\ 6.0\ MF\ 6.4}$ (0.4%) = $ME_{pH\ 7.0\ MF\ 6.0}$ (0.4%) = $ME_{pH\ 6.0\ MF\ 5.0}$ (0.4%) = $ME_{pH\ 7.4\ MF\ 5.0}$ (0.4%) = $ME_{pH\ 4.6\ MF\ 5.0}$ (0.4%) > $ME_{pH\ 5.0\ MF\ 4.0}$ (0.3%) > $ME_{pH\ 7.0\ MF\ 4.0}$ (0.2%). The appearance of a region in a plateau in the mechanical spectrum has been linked to the formation of physical entanglements between polymer molecules that form three-dimensional networks that trap oil droplets (Quintana *et al.*, 2002). Several authors have noted that the properties of the physicochemical emulsions (e.g., rheology, optical properties, stability, molecular partition, and the release characteristics) depend on the properties of the droplets that contain it (Dickinson, 1992; McClements, 2005; Wulff-Pérez *et al.*, 2011).

With respect to the values of the storage modules of different multiple emulsions, $ME_{pH\ 6.0\ MF\ 6.4}$ showed the highest values (312.50 Pa) in the linear viscoelastic region, followed by $ME_{pH\ 6.0\ MF\ 5.0}$ (290.41 Pa) > $ME_{pH\ 7.0\ MF\ 6.0}$ (287.50 Pa) > $ME_{pH\ 6.0\ MF\ 3.6}$ (225.66 Pa) > $ME_{pH\ 5.0\ MF\ 6.0}$ (151.35 Pa) > $ME_{pH\ 7.0\ MF\ 4.0}$ (111.17 Pa) > $ME_{pH\ 7.4\ MF\ 5.0}$ (83.11 Pa) > $ME_{pH\ 4.6\ MF\ 5.0}$ (69.69 Pa) > $ME_{pH\ 5.0\ MF\ 4.0}$ (38.92 Pa). According to the above values, the factor that has the greatest effect on the rheological properties of emulsions was pH.

The loss tangent $\tan\delta$ (G''/G'), is a dimensionless value indicating whether viscous or elastic properties dominate samples (Lobato-Calleros *et al.*, 2009). The values of $\tan\delta$ for all ME in the linear viscoelastic region, showed values lower than 1.0, indicating that the values of G' predominated over G", indicating the domain of elastic character in the linear viscoelastic

region up to a strain of 10%, after this deformation the behavior changed from predominantly elastic to predominantly viscous where $\tan\delta$ values were greater than 1.0.

Conclusion

Multiple emulsions containing NaCl in the internal aqueous phase were stabilized at different MFGM concentrations and different pH levels. In general, those formulated at low concentrations of MFGM exhibited larger droplet sizes ($d_{3,2}$) than those made at higher percentages of MFGM (5%, 6%, and 6.4%). Both, for droplet size and for the release of NaCl from the ME, the factor that showed the greatest effect was the pH. In this work, the viscoelastic properties of ME had a close relationship with its stability and the release kinetics, and indirectly, could be used as an indicator of modulating the pH ratio and concentration of MFGM as necessary to achieve a specific NaCl release rate.

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