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Performance of *Quillaja* bark saponin and β-lactoglobulin mixtures on emulsion formation and stability

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ABSTRACT

The emulsifying properties of a mixture of *Quillaja* bark saponin (QBS) and β-lactoglobulin (β-lg) were evaluated under different pH conditions (7–9) and NaCl concentrations (0–200 mmol·L^{-1}) and compared to the individual components. The formation and stabilization of oil-in-water emulsions were evaluated through visual analysis, droplet size distribution, droplet surface electrical charge (ζ-potential), and emulsion rheology. Both pH and NaCl concentration affected the properties of these emulsions in different ways, depending upon the QBS:β-lg ratio. QBS and/or β-lg emulsions had a relatively high negative droplet charge at pH ranging from 7 to 9 (−76.7 to −17.8 mV), which decreased in magnitude with decreasing pH or with increasing NaCl concentration. All emulsions were polydisperse and presented relatively small average droplet diameters (236–491 nm). Steady-state flow measurements revealed the non-Newtonian, shear-thinning behavior of all emulsions, which was properly described by the Herschel-Bulkley model with a small yield stress (12.9–214.8 mPa) and low apparent viscosity at 100 s^{-1} (1.81–2.97 mPa s). The emulsions were characterized as weak gels by dynamic oscillatory measurements. Most of the emulsions comprising QBS, β-lg, or a mixture of both were stable against droplet coalescence over a pH and NaCl concentration range. Regardless of the tested emulsifier, phase separation did not take place, although droplet creaming was observed. Emulsions comprising QBS and β-lg, both independently and mixed, showed similar emulsifying properties. However, the mixture of these appeared to provide emulsions with improved stability when compared to QBS and β-lg emulsions. The good stability of these emulsions can be attributed to the increased electrostatic repulsion and steric stabilization conferred by the two emulsifiers concurrently.

1. Introduction

Systems containing proteins and low-molecular weight surfactants are present in several food, cosmetic, and pharmaceutical formulations. These compounds comprise the main types of emulsifiers used for such aims, because they are both able to adsorb onto liquid-liquid interfaces, thereby reducing the system's chemical potential. In general, low-molecular weight surfactants are responsible for foaming and emulsification due to their quick interface adsorption, leading to an efficient reduction in the interfacial tension. Proteins, in turn, further stabilize emulsions throughout longer periods because of their ability to form a highly viscoelastic interfacial network surrounding the droplets, which are often electrically charged. Thus, proteins and low-molecular weight surfactants complement each other, synergistically improving the short- and long-term kinetic stabilities of emulsions and foams. This justifies the attention that these emulsifiers have received in the last years (Depree & Savage, 2001; Dickinson & Miller, 2001; Kezwon & Wojciechowski, 2014; Kotsmar et al., 2008; Myers, 1999; Piotrowski, Lewandowska, & Wojciechowski, 2012; Wojciechowski, Kezwon, Lewandowska, & Marcinkowski, 2014; Wojciechowski, Piotrowski, Popielarz, & Sosnowski, 2011).

Several commercially available surfactants are synthetic, most of which are derived from petroleum. However, biosurfactants have been regarded recently as alternatives to synthetic surfactants, especially in the food, pharmaceutical, and cosmetic fields (Kralova & Sjöblom, 2009; Nitschke & Costa, 2007). This approach has been supported by the growing environmental and nutritional

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awareness, in addition to the biocompatibility, biodegradability, and lower toxicity of such compounds (Kitamoto, Isoda, & Nakahara, 2002; Wojciechowski et al., 2011). For this reason, the use of such surfactant class has been currently arousing increased interest from food industries and their consumers.

Quillaja bark saponins (QBS) are biosurfactants extracted from the bark of *Quillaja saponaria* Molina trees (Kezwon & Wojciechowski, 2014; Wojciechowski et al., 2014). Their molecules are chemically made up of a triterpenoid or steroid aglycone moiety attached by glycoside bonds into a linear or branched sugar moiety (Golemanov, Tcholakova, Denkov, Pelan, & Stoyanov, 2012; Hostettmann, 1995; Stanimirova et al., 2011; Wojciechowski, Kezwon, Lewandowska, & Marcinkowski, 2013; Wojciechowski et al., 2011). Quillaja extracts (INS 999) are allowed for human consumption as food additive (emulsifier and foaming agent) according to Codex Alimentarius in the 187 signatory countries, including the United States, United Kingdom, Japan, and China (FAO & WHO, 2016). Therefore, these extracts find commercial applications as emulsifying and foaming agents (Golemanov et al., 2012; Oakenfull, 1981; Yang, Leser, Sher, & McClements, 2013) in both food and cosmetic industries (Golemanov et al., 2012; Kezwon & Wojciechowski, 2014; Stanimirova et al., 2011; Wojciechowski, 2013; Wojciechowski et al., 2014). Saponins have been used either alone or blended with other natural or synthetic surfactants, as well as in combination with food proteins, such as β-lactoglobulin, β-casein and lysozyme (Kezwon & Wojciechowski, 2014), with potential to be used for preparing formulated systems with different biotechnological applications. Such formulations include adjuvants for vaccines and antitumor agents. Moreover, one example of commercial application of this biosurfactant is a Q-Naturale®, a product based on the *Quillaja* saponin extract marketed and approved by FDA for use as emulsifying agent in beverages (FDA, 2016).

β-lactoglobulin (β-lg) is a globular protein that accounts for approximately 45–57 wt% of total whey proteins. As the major fraction of whey protein, β-lg properties tend to be predominant over the properties of products formulated with whey protein concentrates and isolates. β-lg has been extensively studied and structurally characterized (Sawyer & Kontopidis, 2000). Its molecules consist of 162 amino acid residues (18 kDa), out of which five are cysteine residues, as well as high contents of essential amino acids for humans (Cheftel, Cuq, & Lorient, 1992). This protein forms a dimer at pH values between 5 and 8, but undergoes dissociation into monomers at pH values higher than 8 (Verheul, Pedersen, Roefss, & De Kruif, 1999). Each β-lg monomer contains two intramolecular disulfide bonds and a free thiol group (Dickinson, 1998), the latter being remarkably reactive and enabling thiol-disulfide exchange reactions during the conformational changes triggered by thermal treatments and pH variations (Bottomley et al., 1990).

Therefore, both interfacial activity and bulk properties of β-lg and low-molecular weight surfactants mixtures have been characterized (Kezwon & Wojciechowski, 2014; Kotsmar et al., 2008; Piotrowski et al., 2012). Recently, some studies on the interfacial and bulk properties of β-lg and *Quillaja* saponin mixtures have been carried out (Böttcher, Scampicchio, & Drusch, 2016; Kezwon & Wojciechowski, 2014; Piotrowski et al., 2012), although some aspects of emulsions formulated with these mixtures have not been reported to date. To the best of our knowledge, none of the studies addressed the performance of QBS and *β*-lactoglobulin mixtures on emulsion formation and stability at the experimental conditions of the present study (pH = 7, 8 and 9 and NaCl concentration = 0, 100 and 2 mmol·L^{-1}). Hence, in the present study, the emulsifying abilities of a mixture of QBS and β-lg were evaluated in media with these pH values and NaCl concentrations, as these parameters remarkably influence the physical properties of emulsions. Additionally, such properties were compared to those of the individual components. The reported results constitute an additional tool for food developers from both academia and industry, in an effort to create emulsified products with tailored physical characteristics.

2. Materials and methods

2.1. Materials

β-lactoglobulin (β-lg) (90 wt%) was kindly provided by Davisco Foods International Inc. (La Sueur, MN, USA). *Quillaja* bark saponin (QBS), a mixture of triterpene-glycosides extracted from the bark of *Quillaja saponaria* Molina trees, was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The surfactant (active ingredient) content of QBS was reported to be 32 wt%. All concentrations reported in this study were based upon the effective amount of the compound of interest, as disclosed by the suppliers. Commercial sunflower oil (Liza, Cargill Foods, SP, Brazil) was purchased from a local supplier. Analytical-grade sodium chloride, sodium hydroxide, tris(hydroxymethyl)aminomethane, sodium azide, hydrochloric acid, and Sudan III were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All materials were used as received, without further purification. Double distilled, deionized water (electrical resistivity equal to 18.2 MΩ cm; Millipore Inc, Milli-Q, USA) was used in all experiments.

2.2. Preparations of solutions and emulsions

Initially, β-lg (4 mmol·L^{-1}), QBS (4 mmol·L^{-1}), NaCl (1.5 mol·L^{-1}), and tris(hidroximetil)aminometano (tris) (1 mol·L^{-1}) stock solutions were prepared separately by dispersing appropriate amounts of these materials into deionized water containing 0.02 wt% of sodium azide to prevent microbial growth during storage. The stock solutions were stirred for 30 min to ensure complete dispersion and then allowed to rest overnight at 4 °C to ensure complete hydration of the molecules as well as bubble removal. Buffered QBS:β-lg (1:1 ratio) solutions (total concentration of 1.2 mmol·L^{-1}) were prepared in tris buffer (5 mmol·L^{-1}) at different pH values (7, 8 or 9) and NaCl concentrations (0, 100 or 200 mmol·L^{-1}) by mixing suitable volumes of stock solutions. Solutions of the individual components were considered as control in all experiments. The pH of the mixtures was adjusted to the desired value by using 0.1 mol·L^{-1} HCl. The solutions were prepared prior to each experiment. In order to monitor creaming easier, sunflower oil was dyed with a small content of Sudan III, an oil-soluble dye.

Oil-in-water emulsions (10 wt% of sunflower oil and 90 wt% of 1.2 mmol·L^{-1} buffered QBS:β-lg solutions) were prepared using a high-shear mixer (Omni Macro ES Digital Programmable Homogenizer, Kennesaw, USA) equipped with a 20-mm working head. The mixtures were stirred for 3 min at 7500 rpm and at room temperature to yield coarse emulsions. These were then passed through a high-pressure homogenizer (Emulsiflex-C5, Avestin, Ottawa, Canada) 4 times at a pressure of approximately 10,000 psi.

2.3. Zeta potential and particle size measurements

Droplet ζ-potential was estimated from electrophoretic mobility measurements. Average droplet size and emulsion polydispersity index (PDI) were measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instrument Ltd., Worcestershire, UK). Z-average diameter, recorded as a measure of droplet size, as well as PDI, were obtained by Cumulants analysis of correlation function using the initial portion of the data up to 2000 μs? The emulsions were

diluted (at least 1:300) in a 5 mmol·L^{-1} tris buffer with the same pH and NaCl concentration prior to the measurements, aiming to avoid multiple scattering effects (Yang et al., 2013). Buffers were previously filtered using a 0.45 μm cellulose acetate membrane (Millipore) in order to remove undesirable particles and impurities. All measurements were performed right after the homogenization process as well as after 7 days of storage at 25 °C. For the latter, samples were carefully (with no turbulence) collected from the top, middle, and bottom portions of the flasks (0, 1, and 2 cm below the surface, respectively), which had been kept vertically. All measurements were carried out at 25 °C. The results were reported as average values ± standard deviations corresponding to the values obtained for two samples with at least three readings per sample. The ζ-potential measurements of the aqueous phase (buffered QBS:β-lg solutions) were also carried out as previously described, but without dilution.

2.4. Rheological measurements

Rheological studies of the emulsions were carried out using a modular advanced rheometer system (Haake Mars II, Thermo Electron Corp., Karlsruhe, Germany) equipped with a thermostatic bath (Phoenix 2C30P, Thermo Electron Corp., Germany). Samples were obtained immediately after the homogenization process and placed in a stainless steel cone-plate measuring unit (1°; 60 mm diameter; 52 μm gap). Steady-shear flow and oscillatory measurements were performed in duplicates and at a constant temperature (25.0 ± 0.1 °C). Samples were allowed to relax and equilibrate temperature for 5 min prior to the measurements. Fresh samples were used in each assay because of the concerns associated with potential droplet rupture and coalescence during shear. Flow curves were obtained using an up-down-up cycle of shear rates ranging from 10 to 300 s^{-1}, each step taking 2 min. The rheological data of the emulsions was modeled according to the Herschel-Bulkley model presented in Equation (1):

$$\tau = \tau_0 + k\dot{\gamma}^n \quad (1)$$

where τ is the shear stress (Pa), τ_0 is the yield stress (Pa), k is the consistency coeficient (Pa·s^n), $\dot{\gamma}$ is the shear rate (s^{-1}), and n is the flow behavior index (dimensionless).

Dynamic oscillatory assays were also undertaken to determine the storage modulus (G′), loss modulus (G″) and loss tangent (tan δ = G"/G') over an ascending frequency ramp from 0.1 to 3.16 Hz. At frequencies greater than 3.16 Hz, samples exhibited wall slip effects (probably due to the too intense shear, which can induce phase separation). All measurements were performed within the linear viscoelastic range (LVR) (strain amplitude controlled in 0.3%) that had been previously determined from a strain sweep (0.1–100%) and at a constant frequency of 1 Hz.

2.5. Emulsions kinetic stability assessment

The chronological stability of the emulsions against phase separation was visually assessed. As soon as the homogenization process finished, aliquots (5 mL) of fresh emulsions were placed in graduated glass test tubes (125-mm high and with 12 mm of internal diameter), which were then tightly sealed with a plastic film and stored at 25 °C. Phase separation in samples was visually determined by measuring the change in thickness of the cream and serum (a turbid or transparent bottom layer) phases after storage. The extent to which phase separated was quantitatively expressed by a phase separation index (*PSI*), defined as:

$$PSI\ (\%) = 100 \times \frac{V_s}{V_i} \quad (2)$$

In Equation (2), V_s and V_i represent the volume of clear serum formed at the bottom of the tubes and the initial volume of the emulsion, respectively.

Samples (3 mL) were also stored in capped glass flasks for subsequent measurement of droplet size after 7 days. Pictures of these emulsions samples were taken after 7 days using a digital camera (Cyber-shot DSC-W610 14.1 MP, Sony Corp, Tokyo, Japan) in order to compare their appearance changes throughout storage.

2.6. Experimental design and statistical analysis

A three-level full factorial design was carried out to investigate the effects of different pH values (7, 8, and 9) and NaCl concentrations (0, 100, and 200 mmol·L^{-1}) on the formation and kinetic stability of model oil-in-water emulsions formulated with mixtures of QBS and β-lg. The experiments were performed randomly, with two repetitions each. All measurements were reported as mean values ± standard deviations. Data were analyzed by analysis of variance (ANOVA) using the SAS® software, version 9.0 (SAS Institute Inc., NC, USA) licensed for use by the Federal University of Viçosa. Significant differences among the mean values were determined by Tukey's test and, when the parameters were studied in a time course, by paired *t*-test. The suitable fitting of the Herschel-Bulkley model to the experimental data was assessed in terms of the coefficient of determination (R^2) as well as the absolute mean percentage error (*AMPE*), described in Equation (3):

$$AMPE = \frac{100}{n}\sum_{i=1}^{n}\left|\frac{\left(Y_i - \widehat{Y}_i\right)}{Y_i}\right| \quad (3)$$

where Y_i is the i_{th} experimental score, $\widehat{Y}_i$ is the i_{th} predicted score, and n is the number of score pairs. Higher R^2 values and lower *AMPE* values indicate a better fitting. The adopted level of significance (p) was 0.05.

3. Results and discussion

3.1. ζ-potential measurements

3.1.1. QBS and β-lg solutions

In order to estimate the electrostatic forces acting on the particle

Table 1
Droplet surface charge (ζ-potential) (mV) for a continuous phase containing different *Quillaja* bark saponin:β-lactoglobulin (QBS:β-lg) ratios, pH values, and NaCl concentrations.

NaCl (mmol·L^{-1})	pH	QBS:β-lg ratio		
		1:0	1:1	0:1
0	7	-10.8 ± 0.4^{cdA}	-12.2 ± 0.1^{cA}	-21.0 ± 0.4^{cB}
0	8	-14.1 ± 1.6^{eA}	-15.4 ± 0.3^{dA}	-22.2 ± 0.1^{dB}
0	9	-17.0 ± 0.0^{fA}	-17.3 ± 0.9^{dA}	-25.5 ± 0.8^{eB}
100	7	-8.1 ± 0.1^{bB}	-5.7 ± 0.2^{aA}	-10.1 ± 0.2^{aC}
100	8	-6.9 ± 0.1^{abA}	-8.3 ± 0.0^{bB}	-12.7 ± 0.1^{bC}
100	9	-11.1 ± 0.0^{dB}	-5.6 ± 0.0^{aA}	-13.0 ± 0.1^{bC}
200	7	-5.7 ± 0.0^{aA}	-6.5 ± 1.1^{abA}	-10.3 ± 0.1^{aB}
200	8	-6.9 ± 0.3^{abA}	-7.6 ± 0.3^{abA}	-10.9 ± 0.4^{aB}
200	9	-8.8 ± 0.1^{bcB}	-6.2 ± 0.5^{abA}	-12.9 ± 0.8^{bC}

Different superscript letters indicate significant difference ($p < 0.05$). Lowercase letters compare the phase composition conditions (pH and NaCl concentration) for the same QBS:β-lg ratio, whereas uppercase letters compare the QBS:β-lg ratios for the same phase continuous condition (pH and NaCl concentration).

surfaces, the ζ-potentials of particles present in the solutions corresponding to the continuous phases of the studied emulsions were measured (Table 1). All solutions had particles with negatively charged surfaces. The highest ζ-potential magnitude was observed at pH 9 and in the absence of NaCl, whereas the lowest magnitude was observed at pH 7 and 8 in emulsions containing 200 mmol·L^{-1} NaCl ($p < 0.05$).

The net negative charge of QBS can be attributed to the dissociation of −COOH groups presents in its structure, provided that carboxylic groups typically have pK_a values around 3.5 (Yang et al., 2013). For instance, the glucuronic acid group is one of the sugars present in the glycone portion of the saponin molecule and its pK_a is 3.18 (Mitra & Dungan, 1997). According to the results found by Wojciechowski (2013), who studied the surface activity of QBS at the air/water and oil/water interfaces through acid-base titration of QBS supplied by Sigma®, this compound has an estimated molar fraction of proton-dissociable groups equal to 0.5. Also, it behaves like a weak acid, with pK_a = 6.1, which was confirmed by the negative ζ-potential values found in present study.

Similarly, β-lg showed negative charge at all the conditions evaluated, since the tested pH range was higher than that corresponding to the protein isoelectric point (pI = 5.1 (Cheftel et al., 1992)). Therefore, the ζ-potential magnitude increased with increasing pH values, while decreased with increasing NaCl concentrations ($p < 0.05$) because of the electrostatic screening effect caused by the added counter ions that surround the molecules (Hunter & White, 1987; McClements, 2004). The same observation is valid for the QBS particles and β-lg/QBS mixtures. Furthermore, it is also likely that the other components contained in the extract contribute to the net charge since a saponin used is a semi-purified extract.

The fact that the net electrical charge of the mixed solutions at each pH and NaCl concentration (Table 1) has not been proportional to the QBS:β-lg ratio stands out as a possible evidence of interactions between QBS and β-lg. Because of the electrostatic repulsion, hydrophobic interactions may take place between QBS and β-lg. Indeed, β-lg is capable of developing ionic and hydrophobic interactions. Likewise, QBS presents ionic and hydrophobic domains in its structure. Kezwon and Wojciechowski (2014) studied the interactions between QBS from two commercial sources with food proteins (hen egg lysozyme, bovine β-lg, and β-casein). The authors found that the presence of net negative charge over the QBS and β-lg molecules is not enough to prevent attraction between them. In addition, the formation of complexes between QBS and β-lg has been reported (Gee et al., 1997; Piotrowski et al., 2012; Shimoyamada, Ootsubo, Naruse, & Watanabe, 2000) even when both the QBS and the β-lg molecules are negatively charged, which further corroborates this statement.

3.1.2. Electrical charge of emulsion droplets

The ζ-potential results of QBS:β-lg emulsion droplets at different pH values (7–9) and NaCl concentrations (0–200 mmol·L^{-1}) are shown in Table 2. All emulsion droplets exhibited negative ζ-potentials (QBS emulsions: from −21.8 to −86.6 mV, QBS:β-lg (1:1) emulsions: between −20.0 and −79.6 mV, and β-lg emulsions: from −18.8 to −66.4 mV). These values were greater in magnitude than those found for their continuous phases (QBS solutions: between −5.7 and −17.0 mV, QBS:β-lg (1:1) solutions: from −5.6 to −17.3 mV, and β-lg solutions: between −10.1 and −25.5 mV). This difference is because ζ-potential is a function of particle surface charge, of any layer adsorbed at the interface, and of the nature and composition of the surrounding medium in which the particle is suspended.

The ζ-potential value of NaCl-free β-lg emulsions at pH 7 (−54.5 mV) was very close to that reported by Sarkar, Goh, Singh, and Singh (2009) (−57 mV) and Losso and Nakai (2002) (−50.3 mV) for emulsions stabilized by 1 wt% of β-lg at the same pH. On the other hand, NaCl-free QBS emulsions exhibited, at pH values ranging from 7 to 9, more charged surfaces than the emulsions comprising 1 wt% of Q-naturale®, a food ingredient based on *Quillaja* saponin extract (Yang et al., 2013). These difference may be a result of the huge variability in the compositions of different *Quillaja* bark extracts, which contain more than 60 different saponins (van Setten, Jan ten Hove, Wiertz, Kamerling, & van de Werken, 1998) and exhibit different physicochemical properties depending upon the extraction and purification methods (Wojciechowski, 2013). Wojciechowski (2013) verified that, despite being extracted from the same source (bark of the *Quillaja saponaria* Molina tree), two commercial QBS extracts showed markedly different ionic characters, as revealed by acid-base titration.

Table 2
Droplet surface charge (ζ-potential) (mV) for emulsions containing different *Quillaja* bark saponin:β-lactoglobulin (QBS:β-lg) ratio, pH and NaCl concentration, and analyzed directly after preparation.

NaCl (mmol.L^{-1})	pH	QBS:β-lg ratio		
		1:0	1:1	0:1
0	7	-76.7 ± 0.1^{Bc}	-76.4 ± 0.4^{Be}	-54.5 ± 0.0^{Ad}
0	8	-81.0 ± 0.6^{Bd}	-79.4 ± 0.0^{Bf}	-61.3 ± 0.4^{Ae}
0	9	-86.6 ± 0.7^{Ce}	-79.6 ± 0.1^{Bf}	-66.4 ± 0.8^{Af}
100	7	-29.7 ± 0.4^{Cb}	-27.7 ± 0.4^{Bc}	-21.5 ± 0.5^{Ab}
100	8	-29.3 ± 0.3^{Bb}	-28.9 ± 0.6^{Bcd}	-23.9 ± 0.5^{Ab}
100	9	-29.2 ± 0.2^{ABb}	-30.1 ± 0.9^{Bd}	-26.9 ± 0.6^{Ac}
200	7	-21.8 ± 0.4^{Ba}	-20.0 ± 1.1^{ABa}	-18.1 ± 0.1^{Aa}
200	8	-21.4 ± 0.1^{Aba}	-22.4 ± 0.2^{Bb}	-17.8 ± 1.7^{Aa}
200	9	-22.2 ± 0.4^{Aa}	-24.2 ± 0.3^{Bb}	-21.5 ± 0.4^{Ab}

Different superscript letters indicate significant difference ($p < 0.05$). Lowercase letters compare the emulsion compositions (pH and NaCl concentration) for the same QBS:β-lg ratio, while uppercase letters compare the QBS:β-lg mixing ratios for the same emulsion composition (pH and NaCl concentration).

Droplet surface charge was significantly influenced by both pH and NaCl concentration ($p < 0.05$) (Table 2). Regardless of the QBS:β-lg ratio, higher droplet charges were found for NaCl-free emulsions (QBS emulsions: from −76.7 to −86.6 mV, QBS:β-lg (1:1) emulsions: between −76.4 and −79.6 mV, and β-lg emulsions: from −54.5 to −66.4 mV). The ζ-potential values decreased considerably in magnitude with increasing NaCl concentrations up to 100 mmol·L^{-1} (QBS emulsions: between −29.2 and −29.7 mV, QBS:β-lg (1:1) emulsions: from −27.7–30.1 mV, and β-lg emulsions: between −21.5 and −26.9 mV). The addition of 200 mmol·L^{-1} of NaCl led to even more pronounced reductions (QBS emulsions: from −21.4–22.2 mV, QBS:β-lg (1:1) emulsions: between −20.0 and 24.2 mV, and β-lg emulsions: from −17.8 to −21.5 mV). This outcome may be attributed to the increase in the Na^+ content, which binds to the anionic groups present in the molecules that are adsorbed onto droplet surface.

Conversely, for all QBS:β-lg ratios, the dependence of ζ-potential on pH was contrary to that on NaCl concentration: in general, increased pH values led to increased ζ-potential magnitude at a given NaCl concentration (Table 2). The slightly larger surface charge found in more alkaline conditions is associated with the higher degree of deprotonation of the carboxylic groups of protein and surfactant molecules. Higher surface charge was observed at lower NaCl concentration and higher pH levels while lower surface charge was obtained at a higher salt concentration and lower pH levels.

The QBS:β-lg ratio significantly affected ($p < 0.05$) the ζ-potential of the emulsions. At large, higher ζ-potential values were obtained for emulsions stabilized by QBS solely (−81.0 and −86.6 mV at pH 8 and 9, respectively, in the absence of NaCl), whereas smaller values were obtained for emulsions stabilized only

Table 3
Average droplet size (z-average) and polydispersity index (PDI) for emulsions stabilized by different *Quillaja* bark saponin:β-lactoglobulin (QBS:β-lg) ratios at different pH values and NaCl concentrations and analyzed right after preparation.

Continuous phase composition		QBS:β-lg ratio					
		1:0		1:1		0:1	
NaCl (mmol·L^{-1})	pH	z-average (nm)	PDI	z- average (nm)	PDI	z- average (nm)	PDI
0	7	289±3^{Abc}	0.405 ± 0.026^{Aabc}	323 ± 80^{Aa}	0.507 ± 0.141^{Aa}	344 ± 39^{Aab}	0.426 ± 0.009^{Aa}
0	8	275 ± 39^{Abc}	0.371 ± 0.076^{Abc}	283±8^{Aa}	0.368 ± 0.016^{Aa}	282 ± 22^{Ab}	0.389 ± 0.035^{Aa}
0	9	236±1^{Bc}	0.300 ± 0.036^{Bc}	246±4^{Ba}	0.325 ± 0.002^{ABa}	335 ± 13^{Aab}	0.514 ± 0.076^{Aa}
100	7	345 ± 16^{Aabc}	0.460 ± 0.018^{Aab}	277 ± 37^{Aa}	0.386 ± 0.023^{Aa}	289±3^{Aab}	0.476 ± 0.068^{Aa}
100	8	387 ± 37^{Aab}	0.518 ± 0.008^{Aa}	293±6^{Aa}	0.498 ± 0.061^{Aa}	279 ± 45^{Ab}	0.357 ± 0.123^{Aa}
100	9	302 ± 39^{Bbc}	0.393 ± 0.020^{Babc}	246±4^{Ba}	0.381 ± 0.050^{Ba}	491 ± 20^{Aa}	0.536 ± 0.016^{Aa}
200	7	273±6^{Bc}	0.355 ± 0.013^{Abc}	265 ± 19^{Ba}	0.322 ± 0.057^{Aa}	462 ± 71^{Aab}	0.512 ± 0.062^{Aa}
200	8	416 ± 52^{Aa}	0.498 ± 0.001^{Aab}	274±5^{Aa}	0.376 ± 0.022^{Aa}	355 ± 41^{Aab}	0.434 ± 0.046^{Aa}
200	9	282±9^{Abc}	0.354 ± 0.058^{Ac}	261 ± 12^{Aa}	0.314 ± 0.025^{Aa}	320 ± 36^{Aab}	0.373 ± 0.136^{Aa}

Different superscript letters indicate significant difference ($p < 0.05$). Lowercase letters compare the emulsion compositions (pH and NaCl concentration) for the same QBS:β-lg mixing, whereas uppercase letters compare the QBS:β-lg ratios for the same emulsion composition (pH and NaCl concentration).

by β-lg (−17.8 and −18.1 mV at pH 7 and 8, respectively, both containing 200 mmol·L^{-1} of NaCl). Most of the mixed emulsions exhibited droplet charges statistically similar to those comprised in QBS emulsions under the same pH and NaCl concentration.

3.2. Emulsion droplet size

One of the methods intended to evaluate emulsification processes consists of droplet size measurements (Walstra, 1993). Droplet size, in turn, relies upon the balance between two opposing events that occur simultaneously during the homogenization process: droplet breakup and recoalescence (Jafari, Beheshti, & Assadpoor, 2012; Jafari, He, & Bhandari, 2007; McClements, 2004). Furthermore, both droplet size and ζ-potential play a key role in the kinetic stability of the emulsions, since these factors affect several destabilization mechanisms, namely: gravitational separation and droplet aggregation, which significantly increase the droplet size, although the presence of net surface charge results in electrostatic repulsion that may prevent flocculation and coalescence processes.

The intensity size distributions of QBS and β-lg by themselves as well as their mixture presented multimodal distributions regardless of pH and NaCl concentration (supplementary data). The average droplet diameter (z-average) and polydispersity index (PDI) for the emulsions are presented in Table 3. The emulsions exhibited substantial polydispersity and relatively small droplets (z-average < 500 nm), indicating that both pure solutions and β-lg/QBS mixture (1:1) were effective in producing small droplets under the evaluated conditions.

Although the pH and NaCl concentration, as well as the interaction of both, had effects on average droplet size of almost emulsions, these effects did not show any simple variation trend, since several compositions (combinations of pH and NaCl concentration) had statistically similar average droplet diameters (Table 3).

In general, for QBS emulsions, larger and smaller droplets (416 nm for 200 mmol·L^{-1} of NaCl at pH 8; 236 nm for pH 9 without NaCl) were observed in emulsions that showed lower and greater ζ-potential magnitudes (−21.4 and −86.6 mV), respectively. These results indicate that the formation of smaller droplets by QBS may have been enabled by the greater surface droplet charge, provided that the stronger electrostatic repulsive forces may have lessened the recoalescence of oil droplets during homogenization.

Oppositely, smaller droplets did not necessarily show greater ζ-potential magnitude in β-lg emulsions. Some of the emulsions made up of smaller droplets exhibited lower surface charge, presenting |ζ-potential| < 20 mV, condition that indicates low electrostatic stability (Müller, Nitzsche, & Paulke, 1996). This result suggests that other properties may have favored the formation of smaller droplets by β-lg, such as the formation of viscoelastic interfaces and steric repulsion force, which are common for interfaces coated with proteins and that act as a physical barrier against droplet coalescence (McClements, 2004).

Neither pH nor NaCl concentration showed significant effect ($p > 0.05$) on polydispersity index, except for emulsions stabilized by QBS exclusively. For these emulsions, higher PDI values were observed in formulations containing NaCl at pH 8, while lower PDI was shown by NaCl-free emulsions at pH 9. On the whole, the polydispersity degree of QBS emulsions seems to be related to droplet size and surface charge because less polydisperse emulsions were found to show higher droplet charge (in magnitude) and lower average droplet size, whereas the most polydisperse emulsions exhibited lower electrostatic repulsion between the droplets as well as larger average droplet size (Tables 2 and 3).

The mixing ratio between QBS and β-lg had a significant effect ($p < 0.05$) on the average droplet size and PDI only for certain pH and NaCl concentration combinations, namely: pH 9 without NaCl or with 100 mmol·L^{-1} of NaCl, for PDI and droplet size; pH 7 with 200 mmol·L^{-1} of NaCl, for droplet size only. For these conditions, the droplet size and PDI of β-lg emulsions were statistically higher ($p < 0.05$) than those obtained for QBS and mixed emulsions, which did not significantly differ from each other ($p > 0.05$). Higher droplet size and PDI of these β-lg emulsions are likely to be associated with droplets having lower ζ-potential magnitudes when compared to those comprised in QBS emulsions (Table 2).

These results indicate that both QBS and β-lg, as well as the mixture of both, showed similar emulsifying capacities as they produced emulsions with statistically equal average droplet size in most of the evaluated combinations of pH and NaCl concentration (Table 3). However, for the other aforementioned combinations, QBS and its mixture with β-lg led to the formation of smaller droplets when compared to pure β-lg, indicating a greater emulsifying capacity of QBS and of the mixture at these conditions. This result also indicates a synergistic interaction between QBS and β-lg, enhancing their emulsifying properties at such pH and NaCl concentration conditions.

3.3. Rheological properties of the emulsions

In all cases, the Herschel-Bulkley model was well fitted to the experimental data ($R^2 > 99\%$ and $AMPE < 12\%$). The estimated regression parameters of this model are presented in Table 4, along

Table 4
Rheological parameters obtained from the Herschel-Bulkley model (consistency index *k*, flow behavior index *n*, and yield stress τ_0) and apparent viscosity at 100 s^{-1} (η_{100}) for emulsions analyzed directly after preparation.

QBS:β-lg ratio	NaCl (mmol·L^{-1})	pH	η_{100} (mPa·s)	***k*** (mPa·s^n)	***n***	τ_0 (mPa)
1:0	0	7	2.13 ± 0.13^{Ac}	1.85 ± 0.07^{Ba}	0.94 ± 0.00^{Aa}	77.2 ± 3.4^{Ba}
	0	8	2.64 ± 0.10^{Aab}	2.26 ± 0.33^{Aa}	0.91 ± 0.02^{Aa}	118.8 ± 15.4^{Aa}
	0	9	2.70 ± 0.13^{Aab}	2.03 ± 0.01^{Ba}	0.92 ± 0.64^{Aa}	131.1 ± 15.1^{Aa}
	100	7	2.97 ± 0.02^{Aa}	2.00 ± 0.00^{Aa}	0.94 ± 0.04^{Aa}	143.4 ± 4.2^{Aa}
	100	8	2.32 ± 0.15^{Abc}	1.72 ± 0.07^{Ca}	0.95 ± 0.02^{Aa}	98.5 ± 8.4^{Aa}
	100	9	2.12 ± 0.02^{Bc}	1.79 ± 0.32^{Ba}	0.95 ± 0.02^{Aa}	87.5 ± 7.4^{Aa}
	200	7	2.41 ± 0.27^{Abc}	2.22 ± 0.04^{Aa}	0.91 ± 0.01^{Aa}	115.2 ± 44.1^{Aa}
	200	8	2.59 ± 0.00^{Aabc}	1.58 ± 0.49^{Ba}	0.94 ± 0.01^{Aba}	131.3 ± 2.3^{Aa}
	200	9	2.51 ± 0.00^{Aabc}	1.94 ± 0.03^{Ba}	0.93 ± 0.01^{Aa}	109.7 ± 10.7^{Aa}
1:1	0	7	2.03 ± 0.37^{Aa}	5.46 ± 0.57^{Aa}	0.77 ± 0.02^{Bd}	214.8 ± 1.3^{Aa}
	0	8	2.40 ± 0.01^{Aa}	2.63 ± 0.14^{Ab}	0.89 ± 0.01^{Ac}	96.4 ± 18.4^{Ab}
	0	9	2.13 ± 0.12^{Ba}	2.26 ± 0.30^{Bb}	0.92 ± 0.03^{Aabc}	69.4 ± 7.6^{Bbc}
	100	7	2.02 ± 0.18^{Ba}	2.10 ± 0.02^{Ab}	0.93 ± 0.00^{Aabc}	51.1 ± 14.0^{Bc}
	100	8	2.09 ± 0.23^{Aa}	2.36 ± 0.12^{Bb}	0.91 ± 0.01^{Abc}	62.5 ± 18.9^{Abc}
	100	9	1.96 ± 0.02^{Ca}	2.04 ± 0.08^{Bb}	0.94 ± 0.01^{ABab}	51.0 ± 12.0^{Bc}
	200	7	1.98 ± 0.00^{Aa}	2.57 ± 0.37^{Ab}	0.91 ± 0.01^{Abc}	52.3 ± 1.2^{Abc}
	200	8	2.31 ± 0.08^{Ba}	1.90 ± 0.07^{Bb}	0.95 ± 0.02^{Aba}	78.1 ± 8.2^{Bbc}
	200	9	2.03 ± 0.01^{Ba}	2.03 ± 0.12^{Bb}	0.93 ± 0.01^{Aabc}	51.8 ± 3.3^{Bc}
0:1	0	7	1.81 ± 0.06^{Ad}	2.52 ± 0.11^{Bb}	0.90 ± 0.00^{Aab}	24.6 ± 0.6^{Cc}
	0	8	1.96 ± 0.13^{Bcd}	2.85 ± 0.02^{Ab}	0.89 ± 0.01^{Aabc}	29.4 ± 7.4^{Bc}
	0	9	2.11 ± 0.03^{Bcd}	2.99 ± 0.23^{Ab}	0.88 ± 0.01^{Babc}	36.5 ± 11.9^{Bbc}
	100	7	1.94 ± 0.18^{Bcd}	2.53 ± 0.23^{Ab}	0.91 ± 0.01^{Aa}	28.4 ± 10.1^{Bc}
	100	8	2.24 ± 0.13^{Abc}	3.59 ± 0.22^{Aab}	0.85 ± 0.01^{Bbc}	37.6 ± 2.1^{Bbc}
	100	9	2.64 ± 0.01^{Aa}	4.25 ± 0.82^{Aa}	0.84 ± 0.03^{Bc}	72.9 ± 1.5^{Aa}
	200	7	2.00 ± 0.08^{Acd}	2.72 ± 0.09^{Ab}	0.92 ± 0.01^{Aa}	12.9 ± 3.9^{Ac}
	200	8	2.20 ± 0.06^{Bbc}	3.16 ± 0.04^{Aab}	0.89 ± 0.00^{Babc}	37.3 ± 2.5^{Cbc}
	200	9	2.54 ± 0.04^{Aab}	3.32 ± 0.05^{Aab}	0.88 ± 0.01^{Babc}	58.6 ± 11.1^{Bab}

Different superscript letters indicate significant difference ($p < 0.05$). Lowercase letters compare the emulsion compositions (pH and NaCl concentration) for the same QBS:β-lg ratio, whereas uppercase letters compare the QBS:β-lg mixing ratios for the same emulsion composition (pH and NaCl concentration).

with the apparent viscosity at a shear rate of 100 s^{-1} (η_{100}). This shear rate was chosen because it is typical of process such as pumping, stirring, and chewing (McClements, 2004; Steffe, 1996).

All emulsions showed a pseudoplastic behavior ($0.77 < n < 0.95$), which is typical of weak associative interactions (e.g., van der Waals interactions) and suggests the formation of a weak droplet network structure. Several reasons may lead to pseudoplasticity, such as changes in the spatial distribution of the droplets due to the application of shear, removal of solvent molecules originally bound to the droplets, alignment of non-spherical droplets, and deformation and rupture of flakes (Hunter, 1993).

For β-lg emulsions, if NaCl concentration is kept constant, an increase in pH (7–9) led to a decrease in *n* values (0.90–0.88, 0.91 to 0.84, and 0.92 to 0.88 for 0, 100, and 200 mmol·L^{-1} NaCl, respectively), while no simple trend was observed for the changes in NaCl concentration (mean values equal to 0.91, 0.88, and 0.87 for pH 7, 8, and 9, respectively). For the mixed emulsions, there was no obvious trend of changes in *n* values with pH increases, although higher pseudoplasticity (i.e., lower *n*) was observed for NaCl-free emulsions (0.77–0.92). The *n* values of QBS emulsions, in turn, were influenced by neither ($p > 0.05$) pH nor NaCl concentration (mean value = 0.93). At most evaluated conditions of pH and NaCl concentration, the mixing ratio of β-lg and QBS had a significant effect ($p < 0.05$) on *n* values. In general, QBS emulsions exhibited lower shear-thinning (higher *n* values), while β-lg emulsions presented greater shear-thinning.

The consistency index (*k*) of β-lg and mixed emulsions was significantly influenced ($p < 0.05$) by both pH and NaCl concentration. For β-lg emulsions, the increase in pH (7–9) led to an increase in *k* values (2.52–2.99, 2.53 to 4.25, and 2.72–3.32 mPa·s^n for 0, 100, and 200 mmol·L^{-1} of NaCl, respectively), as well as did NaCl concentration (2.52–2.72, 2.85 to 3.16, and 2.99–3.32 mPa·s^n for pH 7, 8, and 9, respectively), mainly at higher pH values. On the other hand, the effects of the mixed emulsions on consistency were different: NaCl addition and pH increases led to decreased *k* values of these emulsions. The consistency index of QBS emulsions was affected by neither pH nor NaCl concentration ($p > 0.05$) (mean value = 1.96 mPa·s^n). Similarly for the pseudoplasticity, the mixing ratio of QBS and β-lg influenced ($p < 0.05$) the emulsion consistency in most of the evaluated conditions. In this case, QBS and mixed emulsions showed similar consistencies, with β-lg emulsions being more consistent than those ($p < 0.05$). Therefore, it is expected that the β-lg emulsions are less unstable because a greater consistency decreases droplet mobility and, hence, reduces system instability.

The yield stress (τ_0) is a parameter of outmost importance in many industrial processes such as pumping, spreading, and coating. This is because it determines the minimum shear stress needed to trigger flow (Steffe, 1996). $\tau_0 > 0$ can be desirable in some products when particles are expected to remain suspended in the medium. Thus, the ability of a certain amount of yield stress offers resistance to flow at low deformations and may favor the kinetic stability of emulsions. τ_0 values of β-lg and mixed emulsions were significantly influenced by pH, NaCl concentration, and the interaction of these factors ($p < 0.05$). Their effects were similar to those observed for consistency index. Thus, higher τ_0 and *k* values were observed for higher pH values and NaCl concentrations in β-lg emulsions as well as for the NaCl-free mixed emulsions at pH 7. For these emulsions, it is expected a lower droplet mobility and, consequently, a greater kinetic stability. In contrast, the τ_0 values of QBS emulsions were influenced by neither pH nor NaCl concentration ($p > 0.05$). Overall, the mixing ratio of QBS and β-lg affected the τ_0 values ($p < 0.05$). The highest and lowest τ_0 values were observed in single QBS and β-lg emulsions, respectively (Table 4).

Apparent viscosity data at 100 s^{-1} (η_{100}) are also shown in Table 4. Only β-lg emulsions had their apparent viscosities affected by both pH and NaCl concentration ($p < 0.05$). For these emulsions, changes similar to those exhibited for yield stress and consistency index were observed. Therefore, greater values for these

parameters were observed at pH 9 with NaCl addition (2.64 and 2.54 mPa·s for 100 and 200 mmol·L^{-1}, respectively), indicating the formation of more structured emulsions. This is in agreement with the mechanical spectra of such emulsions.

Emulsions stabilized by either QBS or β-lg exhibited a relationship between viscosity and droplet size: emulsions with smaller droplets exhibited increased viscosity. The increase in viscosity with the decrease in droplet size could be explained by an increase in the hydrodynamic interactions between neighboring droplets given that, for a certain volume fraction of the dispersed phase, the average distance between the droplets decreases with droplet breakup, which is believed to enhance the hydrodynamic interaction (Pal, 2000). Simultaneously, the high viscosity of the emulsion may have favored the formation of smaller droplets by reducing their mobility, limiting the collisions and, consequently, aggregation and recoalescence during homogenization. Similarly to other rheological parameters, η_{100} was influenced by the mixing ratio of QBS and β-lg in most conditions of pH and NaCl concentration. Generally, higher η_{100} values were observed for QBS emulsions.

Small-amplitude oscillatory shear measurement was performed to determine the viscoelastic properties of the emulsions. Their mechanical spectra (Fig. 1) were obtained from frequency sweep performed within the linear viscoelastic range. All emulsions exhibited a predominantly elastic, gel-like behavior all along the studied frequency range (0.1–3.16 Hz). The storage modulus (G′) was greater than the loss modulus (G″), which can be attributed to the formation of a weakly network. For all emulsions, G″ showed only a slightly dependence on frequency (within the range 0.2 Pa < G" < 1.3 Pa for QBS emulsions, 0.1 Pa < G" < 1.6 Pa for mixed emulsions, and 0.0 Pa < G" < 1.8 Pa for β-lg emulsions), while G′ showed a more evident dependence on frequency (within the range 1.3 Pa < G' < 7.6 Pa for QBS emulsions, 0.8 Pa < G' < 9.4 Pa for mixed emulsions, and 0.0 Pa < G' < 6.8 Pa for β-lg emulsions). This gradual increase in G' with increasing oscillatory frequencies indicates that the strong interactions mainly contributing to this modulus needed a long period of time to relax.

Regardless of the QBS:β-lg ratio and of the continuous phase conditions (pH and NaCl concentration), all emulsions showed G' and G" values lower than 10 Pa. Additionally, a greater change in the viscoelastic modulus values was observed for lower QBS:β-lg ratios for all pH values and NaCl concentrations. On the other hand, an increase in the QBS:β-lg ratio resulted in the formation of a much stronger and more elastic structure, since QBS emulsions showed the highest storage modulus. This observation indicates that such emulsions have a more elastic and better structured tridimensional structure than β-lg emulsions due to the more intense associative interactions among the droplets coated by QBS. These results are consistent with those obtained from steady shear measurements for yield stress (Table 4), since higher and lower yield stress values were obtained for pure QBS and β-lg emulsions, respectively. The mixed emulsions exhibited an intermediate behavior (values of viscoelastic moduli and their frequency dependence), being their results closer to the QBS emulsions than to their β-lg counterparts.

The influence of pH and NaCl concentration on G' and G" was less pronounced for increased QBS:β-lg ratios because the viscoelastic modulus values of QBS emulsions were closer to each other than those of β-lg emulsions, which showed a higher variation at different pH values and NaCl concentrations. These results suggest that the microstructure of QBS emulsions was less affected by both pH and NaCl concentration.

It has been stated that kinetically stable emulsions exhibit mechanical spectra with G′ > G″ and moduli curves almost parallel throughout the entire frequency range (Brummer, 2006; Steffe, 1996). Moreover, the stability is enhanced by predominance of G′ modulus at low frequencies, indicating that the emulsions exhibit long-term stability (Mezger, 2006). However, the dependence of G' on the frequency may indicate that some creaming could occur during storage for long periods of time (Mezger, 2006).

3.4. Emulsion stability

Regardless of the QBS:β-lg ratio and aqueous phase condition (pH and NaCl concentration), no emulsions exhibited sign of phase separation ($PSI = 0$) after 7 days of storage at 25 °C (Fig. 2). The presence of a foam layer could be observed in the top of the vials, since foaming is unavoidable during the homogenization process because both QBS and β-lg are known to act as foaming agents as well (Güçlü-Üstündağ & Mazza, 2007; Piotrowski et al., 2012; Yang & McClements, 2013).

The presence of a vertical color gradient was also observed (Fig. 2), being the color slightly more intense on the top. This suggests the occurrence of a destabilization mechanism by creaming. The more intense staining in the top results from the concentration of larger red droplet on the top, which in turn is a consequence of the different creaming speeds of oil droplets (larger droplets tend to move more rapidly to the top), since the emulsions did not show droplet size uniformity as indicated by the high PDI values (Table 3). Therefore, all emulsions exhibited a good visual stability.

Droplet charge and size (Table 5) were also determined after storage to evaluate the kinetic stability of the emulsions. Few of these exhibited significant ($p < 0.05$) changes in ζ-potential after 7 days of storage, which may be a result of conformational changes in the structure of the adsorbed molecules and the layer in general.

Taking the changes in droplet average diameter after storage into account, it would be expected that variations in droplet size, when occurred, were always positive. Surprisingly, though, some variations were negative, indicating that the average droplet size decreased throughout storage. This droplet shrinkage with time did not result from a spontaneous process of splitting of larger droplets. Droplet shrinkage may be possibly attributed to the following assumptions:

(i) larger droplets and possible clusters formed during storage might have creamed, moved to the top of the cuvette, and got off the laser scope. As a consequence, only smaller droplets were detected and measured;
(ii) clusters formed by weakly bound droplets might have partially dissociated prior to the particle size measurements, since samples assessed after storage were more diluted than those evaluated after homogenization;
(iii) because the emulsions were polydisperse, different coalescence rates might have occurred. As mid-size and large droplets coalesce faster than smaller ones, intermediate and large droplets become bulkier and fewer, whereas smaller droplets might have not coalesced and become more numerous compared to the larger droplets (Jafari et al., 2012).

Thus, according to these assumptions, droplet shrinkage throughout storage is related to the mechanisms of instability that may have been favored by the weaker electrostatic repulsion between the droplets, as most of these emulsions exhibited |ζ-potential| < 30 mV (Table 5), this value denoting the threshold for an emulsion to be considered moderately stable.

Droplet shrinkage was also observed in other studies. Dynamic light scattering measurements revealed the shrinkage of tetradecane-in-water emulsion stabilized by QBS and its mixture with β-lg (Piotrowski et al., 2012) as well as of olive oil-in-water emulsions stabilized by QBS and its mixture with β-casein

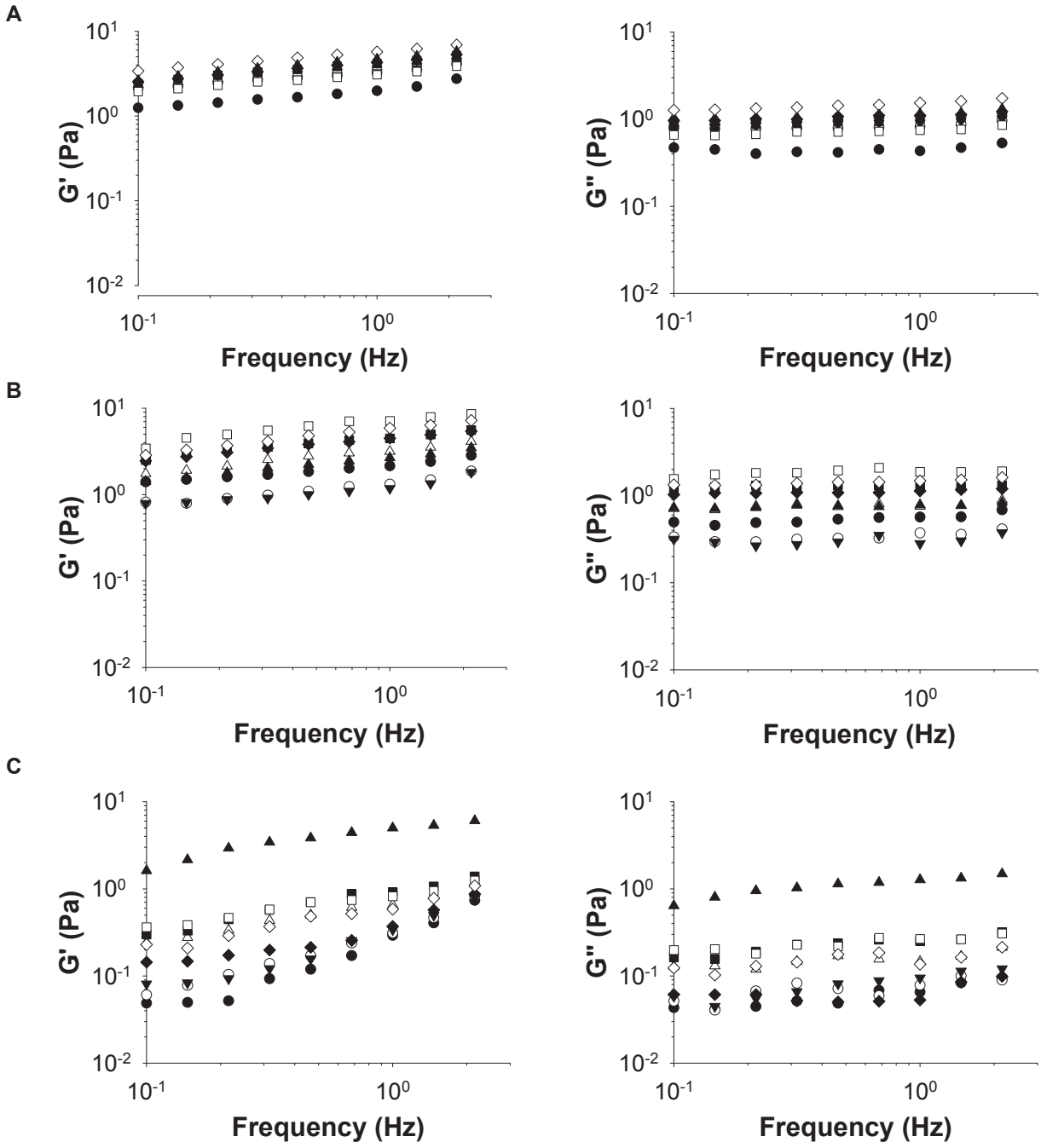

Fig. 1. Elastic modulus (G′) and viscous modulus (G″) of the emulsions stabilized by different *Quillaja* bark saponin:β-lactoglobulin (QBS:β-lg) ratios at different pH values and NaCl concentrations. QBS:β-lg mixing ratios: 1:0 (A), 1:1 (B), and 0:1 (C) at pH 7 and 0 mmol·L^{-1} of NaCl (●), pH 8 and 0 mmol·L^{-1} of NaCl (○), pH 9 and 0 mmol·L^{-1} of NaCl (▼), pH 7 and 100 mmol·L^{-1} of NaCl (Δ), pH 8 and 100 mmol·L^{-1} of NaCl (■), pH 9 and 100 mmol·L^{-1} of NaCl (□), pH 7 and 200 mmol·L^{-1} of NaCl (◆), pH 8 and 200 mmol·L^{-1} of NaCl (◊), and pH 9 and 200 mmol·L^{-1} of NaCl (▲).

(Wojciechowski et al., 2014). Tcholakova, Denkov, Ivanov, and Campbell (2006) reported that the stability of emulsions stabilized by globular milk proteins at pH > 6 and low electrolyte concentration (≤50 mmol·L^{-1}) is mainly governed by long-range electrostatic and van der Waals forces. Under these conditions, the electrostatic repulsion probably keeps the adsorbed protein molecules separated apart and hampers the formation of non-covalent and covalent bonds within the adsorption layers or between the adsorption layers of neighboring droplets (Tcholakova et al., 2006). Thus, emulsion stability does not significantly change with storage time (Tcholakova et al., 2006), as verified in the present study, in which only the diameters of mixed emulsion at pH 9 and 200 mmol·L^{-1} of NaCl and of β-lg emulsion at pH 9 and 100 mmol·L^{-1} of NaCl increased and decreased ($p < 0.05$) after storage, respectively.

Upon NaCl addition, nonetheless, the electrostatic repulsion between droplets probably decreased due to the smaller ζ-potential magnitude (Table 2). According to Tcholakova et al. (2006), at high protein and electrolyte concentrations (≥0.1 wt% and ≥100 mmol·L^{-1}, respectively) and pH > 6.2, the stability of

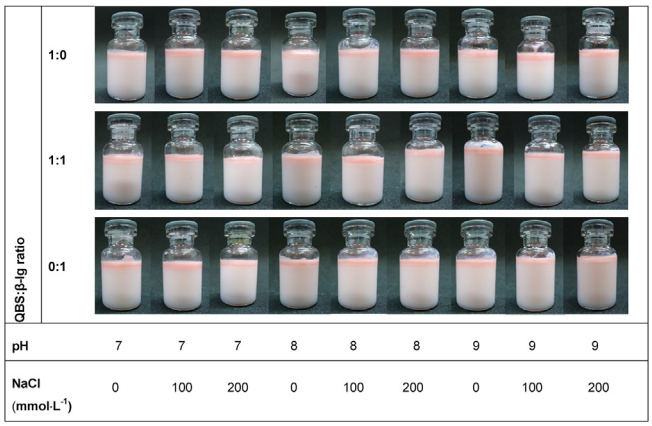

Fig. 2. Visual appearance of *Quillaja* bark saponin:β-lactoglobulin (QBS:β-lg)-stabilized oil-in-water emulsions after storage for 7 days at 25 °C.

emulsions stabilized by globular proteins is related to the steric stabilization afforded by adsorption in overlapping multilayers. However, it should be noted that emulsions stabilized by globular proteins are often resistant to coalescence due to the formation of a strong viscoelastic interface which is able to resist tangential stress that might rupture the interfacial film and cause coalescence (McClements, 2004; Tcholakova et al., 2006). Thus, aggregation and, accordingly, coalescence of droplets stabilized by β-lg were probably prevented by the formation of a strong viscoelastic interface as well as by electrostatic and steric repulsions mainly when the emulsions were formulated without and with the addition of NaCl, respectively.

For QBS emulsions, both electrostatic and steric repulsions played an important role in their stabilization against droplet aggregation (Yang et al., 2013). Yang et al. (2013) studied the formation and stability of O/W emulsions stabilized by *Quillaja* saponin in different conditions (homogenization pressure, number of passes, surfactant concentration, pH, salt concentration, and temperature). These authors reported that, at low salt concentrations, the long-range electrostatic repulsion among the droplets was strong enough to prevent their aggregation while, at high salt concentrations, the long-range attractive interactions (such as van der Waals) overcame the long-range repulsion interactions (such as electrostatic) and led to droplet association. However, the short-range steric repulsion between droplets still seems to be strong enough to prevent coalesce (Yang et al., 2013).

This important contribution of the steric repulsion for the stability of droplets coated by QBS is attributed to the presence of large side groups consisting of sugar molecules extending into the aqueous phase (Ikedo, Shimoyamada, & Watanabe, 1996; Maier, Zeeb, & Weiss, 2014; Stanimirova et al., 2011). It is noteworthy that, although the QBS used in this study does not have high degree of purity (only 32 wt% of saponin present in the extract), it is assumed that saponin is the main responsible for emulsion formation and stability. This observation was corroborated by Stanimirova et al. (2011), who verified the surface properties of unpurified saponin extracts were governed by saponin molecules while other extracted components (such as proteins, polysaccharides, and polyphenols) had secondary importance on these properties.

Mixed emulsions were kinetically stable through the whole pH and NaCl concentration range, except for pH 9 and 200 mmol·L^{-1}, condition in which droplet size increased (Table 5). The stabilization mechanisms provided by the mixed emulsions are most likely the same as that presented by the emulsions stabilized by their pure components (QBS or β-lg). In this case, aggregation was probably prevented by the steric repulsion between the droplets, since both QBS and β-lg show this effect when adsorbed at the interface and, additionally, by the strong electrostatic repulsion between the droplets, mainly when emulsions were formulated in the absence of NaCl (|ζ-potential| > 70 mV).

Overall, the results showed that both emulsions stabilized purely by QBS or β-lg, as well as by their mixture, exhibited good kinetic stability when stored for 7 days at 25 °C, showing no phase separation and, in almost all of the samples, no significant variations in droplet size throughout storage. However, considering the negative droplet size variations as an indication of instability, one may conclude that the mixed emulsions showed higher stability when compared to emulsion stabilized only by QBS or β-lg. This result indicates a synergistic effect between protein and biosurfactant, which were more efficient together in stabilizing O/W emulsions, under the evaluated conditions. Mixed and β-lg emulsions exhibited quite similar characteristics that influence their stabilities (such as droplet size, polydispersity index, and apparent viscosity), which might indicate that slightly stronger electrostatic repulsion between the droplets of mixed emulsions may have been one of the key factors for its higher stability when compared to β-lg emulsions.

4. Conclusion

The present study evaluated the emulsifying properties of QBS:β-lg mixtures (1:1) and compared it with those exhibited by these two components individually, under alkaline conditions (pH 7, 8 or 9) and in the presence of NaCl at low concentrations

Table 5
Average droplet size (z-average), polydispersity index (PDI) and droplet charge (ζ-potential) of emulsions stabilized by different *Quillaja* bark saponin:β-lactoglobulin (QBS:β-lg) ratios at different pH values and NaCl concentrations. Analyzed after storage for 7 days at 25 °C.

NaCl (mmol·L^{-1})	pH	1:0 (QBS:β-lg)			1:1 (QBS:β-lg)			0:1 (QBS:β-lg)		
		z-average (nm)	PDI	ζ (mV)	z- average (nm)	PDI	ζ (mV)	z- average (nm)	PDI	ζ (mV)
0	7	258 ± 4[†]	0.282 ± 0.004	−77.5 ± 0.2	358 ± 9	0.470 ± 0.040	−71.0 ± 0.1[§]	355 ± 65	0.447 ± 0.031	−56.8 ± 1.2
0	8	274 ± 6[†]	0.335 ± 0.009	−80.0 ± 0.6	336 ± 22	0.437 ± 0.008	−72.8 ± 0.2[§]	427 ± 69	0.553 ± 0.021	−61.0 ± 5.2
0	9	330 ± 32	0.411 ± 0.024	−84.8 ± 1.0	334 ± 40	0.431 ± 0.018	−76.3 ± 0.8	322 ± 7[†]	0.417 ± 0.031	−71.9 ± 0.7
100	7	283 ± 7[†]	0.305 ± 0.001	−30.7 ± 0.9	421 ± 119	0.465 ± 0.065	−28.8 ± 0.1	528 ± 7	0.588 ± 0.033	−21.2 ± 0.2
100	8	416 ± 4	0.485 ± 0.019	−29.2 ± 0.1	344 ± 85	0.450 ± 0.073	−29.0 ± 0.1	252 ± 34[†]	0.289 ± 0.095	−29.0 ± 0.3
100	9	311 ± 51	0.376 ± 0.042	−30.6 ± 0.4	315 ± 12	0.425 ± 0.001	−29.6 ± 0.8	320 ± 31[*†]	0.460 ± 0.088	−25.4 ± 0.3
200	7	284 ± 21	0.349 ± 0.052	−21.0 ± 0.6	251 ± 22[†]	0.307 ± 0.069	−20.1 ± 0.3	440 ± 37[†]	0.465 ± 0.004	−18.6 ± 0.7
200	8	425 ± 42	0.448 ± 0.016	−22.0 ± 0.3	439 ± 150	0.490 ± 0.096	−20.0 ± 0.1	358 ± 7	0.414 ± 0.037	−17.9 ± 5.4
200	9	273 ± 12[†]	0.352 ± 0.036	−23.0 ± 0.7	348 ± 16[†]	0.431 ± 0.010	−24.3 ± 0.8	297 ± 3[†]	0.304 ± 0.003	−19.2 ± 0.7

[†] z-average decreased after storage.
[*] z-average that exhibited significant difference after storage according to paired *t*-test ($p < 0.05$).
[§] ζ-potential that exhibited significant difference after storage according to paired *t*-test ($p < 0.05$).

($\leq$200 mmol·L^{-1}). All evaluated conditions led to the formation of polydisperse emulsions, with multimodal droplet size distribution. However, relatively small droplets (average diameter < 500 nm) were formed, which indicate that both solutions were effective in producing small droplets under the evaluated conditions. Regardless of the use of QBS, β-lg or a mixture (1:1) of both, the flow behavior of the emulsions was found to be shear-thinning with small yield stress and low apparent viscosity as well as to show a viscoelastic behavior typical of weak gel. pH and NaCl concentration influenced the physical properties of the emulsions in different ways. Significant effects of these factors were checked for droplet surface charge, droplet size, and apparent viscosity of emulsions stabilized either by QBS or β-lg, separately, as well as for coefficients of Herschel-Bulkley model of β-lg and mixed emulsions. The emulsifier (QBS, β-lg or both) also showed a significant effect on the emulsion properties. In general, higher droplet charge, apparent viscosity, yield stress, and flow behavior index were obtained for emulsions stabilized purely by QBS. Droplet size was not significantly affected by the mixing ratio of QBS and β-lg in most of the evaluated pH and NaCl concentration combinations. The emulsions were stable against phase separation. The droplet size of pretty much all emulsions did not change significantly after 7 days of storage. Mechanisms of steric and electrostatic repulsion proved to be important to provide stability to the different emulsions. When compared, the mixtures of QBS and β-lactoglobulin (1:1) showed similar emulsifying properties to surfactant and protein pure at pH 7–9 and NaCl concentration 0–200 mmol·L^{-1}. However, the mixtures seem improved stability when compared to saponin and protein emulsions, showing promising. The results of this study indicate that saponin can be used in combination with β-lactoglobulin to produce emulsions with improved stability. Furthermore, new surfactant:protein ratios should be evaluated to determine the best proportion for emulsion stabilization purposes, as well as more complex systems (e.g., real foods).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2017.01.013.

References

Böttcher, S., Scampicchio, M., & Drusch, S. (2016). Mixtures of saponins and beta-lactoglobulin differ from classical protein/surfactant-systems at the air-water interface. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 506*, 765–773.

Bottomley, R. C., Evans, M. T. A., & Parkinson, D. J. (1990). Whey proteins. In P. Harris (Ed.), *Food gels* (Vol. 11, pp. 435–466). London/New York: Elsevier Applied Science.

Brummer, R. (2006). *Rheology essentials of cosmetic and food emulsions*. Germany: Springer Berlin Heidelberg.

Cheftel, J.-C., Cuq, J.-L., & Lorient, D. (1992). *Protéines alimentaires biochimie, propriétés fonctionnelles, valeur nutritionnelle, modifications chimiques*. Paris: Technique et documentation-Lavoisier.

Depree, J. A., & Savage, G. P. (2001). Physical and flavour stability of mayonnaise. *Trends in Food Science & Technology, 12*(5–6), 157–163.

Dickinson, E. (1998). Proteins at interfaces and in emulsions Stability, rheology and interactions. *Journal of the Chemical Society, Faraday Transactions, 94*(12), 1657–1669.

Dickinson, E., & Miller, R. (2001). *Food Colloids: Fundamentals of formulation*. Cambridge, UK: Royal Society of Chemistry.

FAO (Food and Agriculture Organization of the United Nations) & WHO (World Health Organization). (2016). *Codex Alimentarius - interantional food Standards: List of Codex speficications for food additives*. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/ru/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCAC%2BMISC%2B6-2015%252FCXA_006efs.pdf (Acessed 20 November 2016).

FDA (Food and Drug Administration). (2016). *Q-naturale 300 (registered as raw material)*. http://www.fda.gov.ph/consumers-corner/registered-food-products/321363-FR-4000000280170 (Acessed 21 December 2016).

Gee, J. M., Wal, J. M., Miller, K., Atkinson, H., Grigoriadou, F., Wijnands, M. V. W., et al. (1997). Effect of saponin on the transmucosal passage of β-lactoglobulin across the proximal small intestine of normal and β-lactoglobulin-sensitised rats. *Toxicology, 117*(2–3), 219–228.

Golemanov, K., Tcholakova, S., Denkov, N., Pelan, E., & Stoyanov, S. D. (2012). Surface shear rheology of saponin adsorption layers. *Langmuir, 28*(33), 12071–12084.

Güçlü-Üstündağ, Ö., & Mazza, G. (2007). Saponins: Properties, applications and processing. *Critical Reviews in Food Science and Nutrition, 47*(3), 231–258.

Hostettmann, K. M. A. (1995). *Saponins*. Cambridge; New York: Cambridge University Press.

Hunter, R. J. (1993). *Introduction to modern colloid science*. Oxford, UK: Oxford University Press.

Hunter, R. J., & White, L. R. (1987). *Foundations of colloid science*. United States: Clarendon Press.

Ikedo, S., Shimoyamada, M., & Watanabe, K. (1996). Interaction between bovine serum albumin and saponin as studied by heat stability and protease digestion. *Journal of Agricultural and Food Chemistry, 44*(3), 792–795.

Jafari, S. M., Beheshti, P., & Assadpoor, E. (2012). Rheological behavior and stability of d-limonene emulsions made by a novel hydrocolloid (Angum gum) compared with Arabic gum. *Journal of Food Engineering, 109*(1), 1–8.

Jafari, S. M., He, Y., & Bhandari, B. (2007). Production of sub-micron emulsions by ultrasound and microfluidization techniques. *Journal of Food Engineering, 82*(4), 478–488.

Kezwon, A., & Wojciechowski, K. (2014). Interaction of Quillaja bark saponins with food-relevant proteins. *Advances in Colloid and Interface Science, 209*(0), 185–195.

Kitamoto, D., Isoda, H., & Nakahara, T. (2002). Functions and potential applications of glycolipid biosurfactants - from energy-saving materials to gene delivery carriers. *Journal of Bioscience and Bioengineering, 94*(3), 187–201.

Kotsmar, C., Grigoriev, D. O., Xu, F., Aksenenko, E. V., Fainerman, V. B., Leser, M. E., et al. (2008). Equilibrium of adsorption of mixed milk protein/surfactant solutions at the water/air interface. *Langmuir, 24*(24), 13977–13984.

Kralova, I., & Sjöblom, J. (2009). Surfactants used in food industry: A review. *Journal of Dispersion Science and Technology, 30*(9), 1363–1383.

Losso, J. N., & Nakai, S. (2002). Stabilization of oil-in-water emulsions by β-Lactoglobulin–Polyethylene glycol conjugates. *Journal of Agricultural and Food Chemistry, 50*(5), 1207–1212.

Maier, C., Zeeb, B., & Weiss, J. (2014). Investigations into aggregate formation with oppositely charged oil-in-water emulsions at different pH values. *Colloids and Surfaces B: Biointerfaces, 117*(0), 368–375.

McClements, D. J. (2004). *Food Emulsions: Principles, practices, and techniques*. United States: Taylor & Francis.

Mezger, T. G. (2006). *The rheology Handbook: For users of rotational and oscillatory Rheometers: Vincentz network*.

Mitra, S., & Dungan, S. R. (1997). Micellar properties of Quillaja saponin. 1. Effects of temperature, salt, and pH on solution properties. *Journal of Agricultural and Food Chemistry, 45*(5), 1587–1595.

Müller, R. H., Nitzsche, R., & Paulke, B. R. (1996). *Zetapotential und Partikelladung in der Laborpraxis: Einführung in die Theorie, praktische Messdurchführung, Dateninterpretation*. Germany: Wiss: Verlag-Ges.

Myers, D. (1999). *Surfaces, interfaces, and colloids: Principles and applications*. Wiley-VCH.

Nitschke, M., & Costa, S. G. V. A. O. (2007). Biosurfactants in food industry. *Trends in Food Science & Technology, 18*(5), 252–259.

Oakenfull, D. (1981). Saponins in food—a review. *Food Chemistry, 7*(1), 19–40.

Pal, R. (2000). Shear viscosity behavior of emulsions of two immiscible liquids. *Journal of Colloid and Interface Science, 225*(2), 359–366.

Piotrowski, M., Lewandowska, J., & Wojciechowski, K. (2012). Biosurfactant–protein Mixtures: Quillaja bark saponin at water/air and water/oil interfaces in presence of β-lactoglobulin. *The Journal of Physical Chemistry B, 116*(16), 4843–4850.

Sarkar, A., Goh, K. K. T., Singh, R. P., & Singh, H. (2009). Behaviour of an oil-in-water emulsion stabilized by β-lactoglobulin in an in vitro gastric model. *Food Hydrocolloids, 23*(6), 1563–1569.

Sawyer, L., & Kontopidis, G. (2000). The core lipocalin, bovine β-lactoglobulin. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1482*(1–2), 136–148.

van Setten, D. C., Jan ten Hove, G., Wiertz, E. J. H. J., Kamerling, J. P., & van de Werken, G. (1998). Multiple-stage tandem mass spectrometry for structural characterization of saponins. *Analytical Chemistry, 70*(20), 4401–4409.

Shimoyamada, M., Ootsubo, R., Naruse, T., & Watanabe, K. (2000). Effects of soybean saponin on protease hydrolyses of β-lactoglobulin and α-lactalbumin. *Bioscience, Biotechnology, and Biochemistry, 64*(4), 891–893.

Stanimirova, R., Marinova, K., Tcholakova, S., Denkov, N. D., Stoyanov, S., & Pelan, E. (2011). Surface rheology of saponin adsorption layers. *Langmuir, 27*(20), 12486–12498.

Steffe, J. F. (1996). *Rheological methods in food process engineering*. USA: Freeman Press.

Tcholakova, S., Denkov, N. D., Ivanov, I. B., & Campbell, B. (2006). Coalescence stability of emulsions containing globular milk proteins. *Advances in Colloid and Interface Science, 123–126*(0), 259–293.

K. G. Verheul, M., Pedersen, J. S., Roefss, S. P., & Kruif, D. (1999). Association behavior of native beta-lactoglobulin *Biopolymers, 49*(1), 11–20
Walstra, P. (1993). Principles of emulsion formation. *Chemical Engineering Science, 48*(2), 333–349.
Wojciechowski, K. (2013). Surface activity of saponin from Quillaja bark at the air/water and oil/water interfaces. *Colloids and Surfaces B: Biointerfaces, 108*(0), 95–102.
Wojciechowski, K., Kezwon, A., Lewandowska, J., & Marcinkowski, K. (2013). Effect of β-casein on surface activity of Quillaja bark saponin at fluid/fluid interfaces. *Food Hydrocolloids*, (0).
Wojciechowski, K., Kezwon, A., Lewandowska, J., & Marcinkowski, K. (2014). Effect of β-casein on surface activity of Quillaja bark saponin at fluid/fluid interfaces. *Food Hydrocolloids, 34*(0), 208–216.
Wojciechowski, K., Piotrowski, M., Popielarz, W., & Sosnowski, T. R. (2011). Short- and mid-term adsorption behaviour of Quillaja Bark Saponin and its mixtures with lysozyme. *Food Hydrocolloids, 25*(4), 687–693.
Yang, Y., Leser, M. E., Sher, A. A., & McClements, D. J. (2013). Formation and stability of emulsions using a natural small molecule surfactant: Quillaja saponin (Q-Naturale®). *Food Hydrocolloids, 30*(2), 589–596.
Yang, Y., & McClements, D. J. (2013). Encapsulation of vitamin E in edible emulsions fabricated using a natural surfactant. *Food Hydrocolloids, 30*(2), 712–720.