Formulation and stabilization of oil-in-water nanoemulsions using a saponins-rich extract from argan oil press-cake

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Abstract

In this study, we formulated and stabilized oil-in-water nanoemulsions using a crude extract from argan press-cake as sole emulsifier. Various extracts from argan press-cake were prepared in order to select the most surface-active one(s) foreseeing emulsions preparation. Fifty percent (v/v) ethanolic extract reduced the interfacial tension to a minimum value at both MCT oil and soybean oil interfaces (12.7 and 10.5 mN m^{-1} respectively). This extract was also effective at producing fine emulsions with small droplet sizes ($d_{3,2}$ < 115 nm) and good physical stability using different oils such as soybean oil, MCT oil and fish oil and at conventional homogenization conditions (100 MPa for 4 passes). On the other hand, the emulsions were very sensitive to NaCl addition (≥25 mM) and to acidic pH (<3) indicating that the main stabilization mechanism is electrostatic, likely due to the presence of surface-active compounds with ionizable groups such as saponins.

Keywords: Argan press-cake, Emulsifier, Interfacial tension, Nanoemulsions, Saponins

1. Introduction

Plant by-products are usually viewed as rich and cheap sources of functional compounds with numerous potential applications. Nevertheless, if not efficiently processed, they can cause adverse effects on the ecosystem and human health (Helkar, Sahoo & Patil, 2016). Therefore, a proper management to turn these residues into healthy and economically rewarding ingredients is of great interest. Indeed, many food industries and laboratories are continuingly trying to explore and valorize these underestimated sources (Barbosa-Pereira, Pocheville, Angulo, Paseiro-Losada, & Cruz, 2013; Morales, Barros, Ramirez-Moreno, Santos-Buelga, & Ferreira, 2015).

Argan (Argania spinosa) is an endemic specie of Morocco. It grows naturally in the region of Souss (south-west) over an area of 830.000 ha, thus covering more than 17% of the Moroccan forest (Charrouf & Guillaume, 2009). The argan tree is of great interest for social, environmental and economic reasons. The wood is used as fuel by locals. The leaves are exploited as a fodder for goats and camels. The long-deep roots are an excellent front-line against erosion and sand encroachment. However, the true value of the argan tree is in its oil (Lybbert, Aboudrare, Chaloud, Magnan, & Nash, 2011). Argan oil is extracted from the fruit kernels and it is famous worldwide for having multiple dietary and cosmetic properties. The paste resulting from its extraction however is mainly used as feed for cattle (Guillaume & Charrouf, 2011). It is most known to be rich in bidesmosidic saponins (Chafchaouni-Moussaoui, Charrouf, & Guillaume, 2013; Charrouf et al., 1992). More recently, various other saponins have been discovered in this by-product, with the majority of them having glucuronic acids in their chemical structure (Henry et al., 2013).

Emulsions are one of the encapsulation and delivery systems in food, cosmetic and pharmaceutical industries (McClements, 2016). An emulsion consists of a dispersed phase, a continuous phase and emulsifier(s) in a homogeneous mixture prepared either through high-energy or low-energy forces (McClements, 2016). In order to improve emulsions stability and properties, many investigations have been done on each of these components. For example, multiple homogenization devices have been developed with some of them already being applied at large-scale in industry (Jafari, He, & Bhandari, 2007). In addition, numerous kinds of synthetic emulsifiers with different physicochemical properties and stabilization mechanisms are currently available. Selecting the right emulsifier depends on many factors, mainly the potential use, formula and the storage conditions of the final product (Ozturk & McClements, 2016).

Recently, there has been an increase interest in replacing synthetic emulsifiers with natural alternatives in order to develop entirely green label products that meet consumers' criteria. Indeed, different types of bio-surfactants have been identified, characterized and successfully used to prepare emulsion-based products (McClements, 2016). Among these, proteins (e.g. caseins, whey proteins), polysaccharides (e.g. starch, pectin), phospholipids (e.g. lecithins) and saponins (Klang & Valenta, 2011; Lam & Nickerson, 2013; Ozturk, Argin, Ozilgen, & McClements, 2014) may be relevant.

More recently, a food-grade emulsifier made from the bark of Quillaja saponaria tree has been successfully used to prepare oil-in-water (O/W) nanoemulsions with narrow droplet size and with improved stability over a wide range of environmental stresses (Yang, Leser, Sher, & McClements, 2013). The active ingredient is believed to be a mixture of small molecular weight

bidesmosidic saponins. Quillaja saponins have been previously reported to be highly surface-active at the oil/water interface thus potentially acting as emulsifiers (Mitra & Dungan, 1997). In a different study, another research group was capable of producing O/W emulsions by ultra-sonication using a saponins-rich extract from ginseng roots (Rosa, Silva, Santos, Petenate, & Meireles, 2016). Based on these findings, saponins appear to be effective at formulating and stabilizing O/W emulsions for multiple applications. The stabilization mechanism is mainly related to the presence of glucuronic acids in their chemical structure, which tend to be highly negatively charged at neutral pH, but progressively loose this charge as the pH is reduced (Ozturk & McClements, 2016). It was therefore suggested to identify other commercially viable sources of such natural emulsifiers.

Following this, the present study evaluated the emulsifying properties of a saponins-rich extract from argan oil press-cake. Our aim was to produce, characterize and stabilize model O/W emulsions using this extract as sole emulsifier.

2. Materials and methods

2.1. Materials

Cold-pressed argan kernels cake was provided by an oil-producing cooperative in the region of Essaouira, Morocco. Ethanol, oleanolic acid standard (97%), bovine serum albumin (BSA), Bradford reagent, refined soybean oil, D- limonene, polyoxyethylene (20) sorbitan monolaurate (Tween 20), sodium azide, sodium chloride, sodium hydroxide and hydrochloric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Refined medium- chain triglyceride (MCT) oil was kindly provided by Taiyo Kagaku Co., Ltd. (Tokyo, Japan). Fish oil was obtained from Kain Co., Ltd. (Taipei, Taiwan). Deionized water (18 MΩ cm) was produced

by a Milli-Q system (Arium® pro, Goettingen, Germany) and used to prepare all solutions and emulsions in the current study.

2.2. Preparation and analysis of extracts

The press-cake was finely grinded (<0.5 mm) and added to fresh mixtures of distilled water and ethanol (0–100%, (v/v)), at a solvent:powder ratio of 10:1. The suspensions were then continuously shaken for 24 h at room temperature until complete whitening of the powders. Finally, after centrifugation (MX-307, Tomy Digital Biology Co., Ltd., Tokyo, Japan) and filtration to remove solid particles, the solvent was evaporated at 40 °C and 16 hPa (Eyela EVP-1100, Shanghai Co., Ltd., China) and the residues were re-dissolved in milli-Q water to prepare stock solutions of appropriate concentration.

Total saponins content was spectrophotometrically determined (V-570, JASCO Co., Hachioji, Japan) according to the method of Xiang, Tang, Chen, and Shi (2001), with minor modifications. Briefly, 0.1 mL of extract solution, 0.1 mL of a freshly prepared 5% vanillin-acetic acid solution and 1.2 mL of 60% perchloric acid were mixed together and incubated at 70 °C for 20 min. After cooling to room temperature, 5 mL of ethyl acetate was then added to the mixture and the absorbance was measured at 550 nm against a blank solution as reference. Oleanolic acid (0–0.1 mL of a 2.4 mg mL^{-1} solution) was used as a standard ($R^2 = 0.9995$).

Total proteins content was determined according to the Bradford's method (Bradford, 1976). Briefly, 3 mL of Bradford reagent was added to 0.1 mL of extract solution and left to incubate for 5 min at room temperature. The absorbance was then measured at 595 nm and the amount of total proteins in extract was expressed as BSA equivalent using a BSA standard curve (0–1.4 mg mL^{-1}).

2.3. Interfacial tension measurements

Initial experiments were carried out in order to study the effect of extract type on the interfacial tension at the oil/water interface. We used a pendant drop method to quantify interfacial tension on a fully automatic interfacial tensiometer (PD-W, Kyowa Interface Science Co., Ltd., Saitama, Japan). Briefly, the extract (1% (w/w)) was placed in a syringe, the oil (MCT oil or soybean oil) inside of a glass cell and a drop was made until it reached its maximum volume. A high-resolution camera captured then the image of the drop and according to its size, shape and to the density difference between the two phases, the software calculated the interfacial tension using the Young-Laplace equation. For the critical micellar concentration (CMC) determination, we selected 50% (v/v) ethanolic extract to measure the interfacial tension between soybean oil and various solutions (0.01–3% (w/w)) of this extract.

2.4. Emulsions preparation

O/W emulsions were prepared by homogenizing appropriate amounts of oil phase (soybean oil, MCT oil, fish oil or D-limonene), containing 0.002% (w/w) of Sudan IV dye, and aqueous phase (0.1–3% (w/w) Tween 20 or 50% (v/v) ethanolic extract). Firstly, a coarse emulsion was obtained by blending the two phases together using a rotor-stator homogenizer (Polytron, PT-3000 Kinematica-AG, Littace, Switzerland) at 7000 rpm for 5 min. This coarse emulsion was then passed through a high-pressure homogenizer (NanoVater, NV200, Yoshida Kikai, Nagoya, Japan) at various pressures (30–130 MPa) and for different number of passes (1–10).

2.5. Stability testing

2.5.1. pH

In this section, emulsions were prepared using phosphate buffer (10 mM). Samples were initially diluted with buffer solutions of appropriate pH (1-10). The pH was then adjusted, if necessary, using either hydrochloric acid (1M) or sodium hydroxide (1M). Samples were finally stored for 24 h at 25 ± 2 °C prior to analysis.

2.5.2. Ionic strength

The ionic strength of prepared emulsions was adjusted to different levels (0-200 mM) by adding appropriate amounts of a NaCl stock solution (1M). Samples were then stored at 25 ± 2 °C and analyzed for particle size changes on the following day.

2.5.3. Long-term storage

Prepared emulsions were incubated at different temperatures (5, 25 or 45 °C) and monitored for particle size changes over a period of two months. Sodium azide (0.02% (w/w)) was added to the emulsions in order to prevent microbial growth during this period.

2.6. Particle characterization

Particle size measurements were conducted using a laser diffraction particle size analyzer (LS 13320, Beckman Coulter, Brea, USA). The refractive indices of soybean oil, MCT oil, fish oil and D-Limonene were 1.432, 1.447, 1.481 and 1.471 respectively. The droplet charge was determined using a ζ-potential analyzer (Zetasizer, Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) over 10 to 100 runs per cycle. The samples were diluted (1:100) with phosphate buffer of appropriate pH prior to analysis in order to prevent multiple scattering effects.

2.7. Statistical analysis

In this study, we used a single factor design in order to evaluate the emulsifying properties of a natural extract from argan press-cake. All experiments were repeated at least twice with no less than three measurements per sample. Additionally, a statistical analysis was performed using Statistix 8.1 software. The measurements were subjected to analysis of variance (ANOVA) using the 'Tukey' test to assess significant differences among variables at 95% confidence level.

3. Results and discussion

3.1. Interfacial properties of aqueous-ethanolic extracts

Argan oil press-cakes are known to contain large amounts of saponins (Chafchaouni-Moussaoui, Charrouf, & Guillaume, 2013). These compounds are also known to have surface-active properties, potentially acting as emulsifiers (Oleszek & Hamed, 2010). Since more than one method for argan saponins extraction are reported (El Fakhar, Charrouf, Coddeville, Leroy, Michalski, & Guillaume, 2007; Henry, Kowalczyk, Maldini, Piacente, Stochmal, & Oleszek, 2013), we evaluated the completeness of our extraction procedure by employing a sequence of increasing polarities of ethanol on different samples (4 g). We thereafter, measured the interfacial tension as a function of extract type in order to select the most surface-active one(s) for subsequent experiments. In general, all extracts were capable of significantly reducing the interfacial tension at both oil types interfaces (Fig. 1a). For example, it was reduced from 26.8 mN m^{-1} to around 12.7 mN m^{-1} when replacing milli-Q water with 1% (w/w) of extract 50% (v/v) ethanol, at the MCT oil interface. This value was further reduced from 26.3 mN m^{-1} to 10.5 mN m^{-1} when soybean oil was used. In the current study, we used refined MCT oil or soybean oil in order to prevent interaction of compounds under investigation with endogenous impurities that might be present in commercial vegetable oils

(Gaonkar, 1989). Therefore, the significant difference in interfacial tension, for the same extract type, at these two oils interfaces can be attributed to viscosity and density differences.

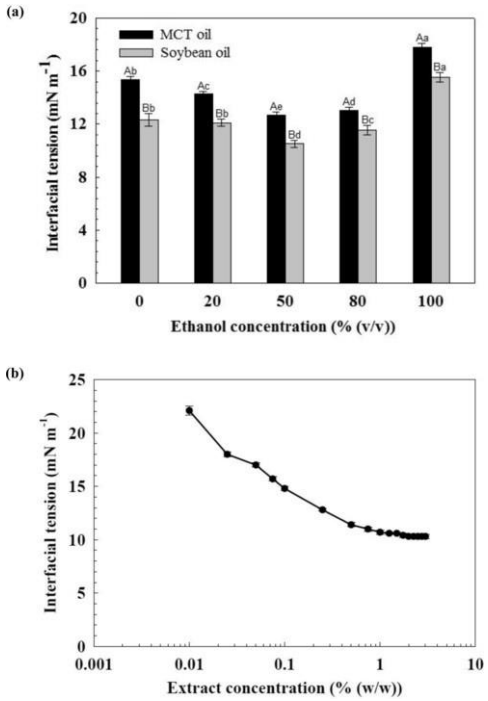

Fig. 1 Effect of the extraction solvent on the interfacial tension of newly created interfaces. (a) Effect of extract type (1% (w/w)) on the interfacial tension at the oil/water interface. (b) Interfacial tension as a function of 50% (v/v) ethanolic extract concentration at the soybean oil/water interface. The interfacial tension between milli-Q water and oil was 26.8 ± 0.2 and 26.3 ± 0.1 for MCT oil and soybean oil respectively.

Interfacial tension of surfactants plays an important role in determining their ability to form and stabilize emulsions (Stamkulov, Mussabekov, Aidarova, & Luckham, 2009). Usually, the lower is the interfacial tension, the greater are the emulsifying properties (Amine, Dreher, Helgason, & Tadros, 2014). For this reason, we selected 50% (v/v) ethanolic extract for the CMC determination and emulsions preparation. Overall, the interfacial tension decreased gradually with increasing this

extract concentration, until it reached a fairly constant value at high levels (Fig.1b). Using the method described previously by Benincasa, Abalos, Oliveira, and Manresa (2004), we estimated the CMC value to be around 0.44% (w/w). To the best of our knowledge, the CMC values of argan press-cake extracts have not been reported before. We therefore prepared our emulsions at various emulsifier concentrations in order to better understand the surface-active behavior of this extract.

3.2. Saponins and proteins contents in aqueous-ethanolic extracts

Saponins and proteins are known to have surface-active properties because of the presence of hydrophilic and lipophilic moieties in their chemical structures (Lam & Nickerson, 2013; Oleszek & Hamed, 2010). Therefore, we specifically targeted these compounds in the prepared extracts in order to investigate the contribution of specific ingredients to the obtained interfacial properties (section 3.1). Overall, total saponins and total proteins contents were significantly affected by extraction solvent mixture.

Fifty percent (v/v) ethanolic extract presented the highest saponins concentration (4.2% (w/w)), followed by 80% (v/v) ethanolic extract, and finally pure ethanol (Fig. 2a). Similar results were previously obtained by Henry, Kowalczyk, Maldini, Piacente, Stochmal, and Oleszek (2013). They found that the use of aqueous 50% (v/v) ethanol improved the extraction of more polar saponins from argan oil press-cake with the majority of them having glucuronic acids residues in their chemical structure. In general, argan press-cakes are known to contain large amounts of saponins (Chafchaouni-Moussaoui, Charrouf, & Guillaume, 2013). However, in almost all former studies, extraction was only performed using high concentrations of ethanol or methanol (Charrouf, Wieruszeski, Fkihtetouani, Leroy, Charrouf, & Fournet, 1992; El Fakhar, Charrouf, Coddeville, Leroy, Michalski, & Guillaume, 2007). The results depicted in Fig. 2a show that, 20% (v/v) ethanol

and water extracts also presented relatively high contents of saponins (3.5 and 2.6% (w/w) respectively), likely due to proper swelling of the plant material during extraction. Such observation was previously reported by Li, Zu, Fu, Yang, Li, Li, et al. (2010), who found that suitable water in solvent could facilitate extraction yields of saponins by enhancing the surface contact area between the plant matrix and the solvent.

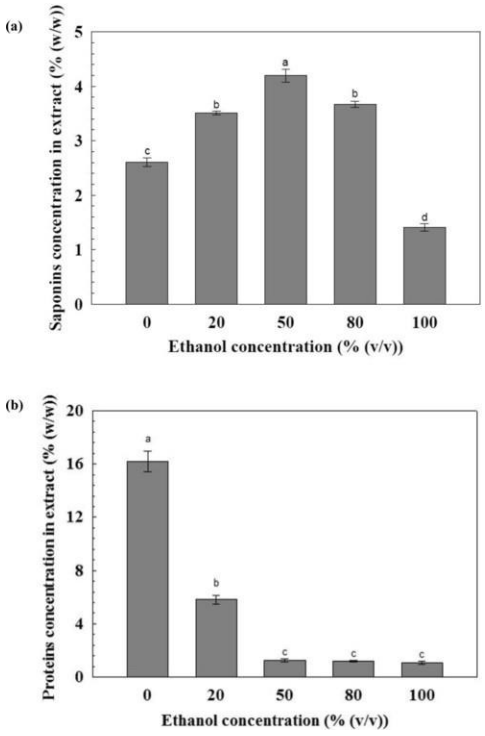

Fig. 2. Effect of ethanol concentration on (a) total saponins and (b) total proteins contents in extracts.

For proteins analysis, it is generally known that ethanol causes proteins precipitation due to structural and conformational modifications (Yoshikawa, Hirano, Arakawa, & Shiraki, 2012). In our results, the increase of ethanol concentration gradually decreased the proteins content until it reached a fairly constant value at high concentrations of ethanol (50-100% (v/v)) (Fig. 2b). To the

best of our knowledge, argan oil press-cakes are mainly recycled for the moment as cattle feed because of their high protein and glucide contents. However, no previous studies have analyzed the proteins profiles of this material. We therefore attributed the constant concentration (~1% (w/w)) of proteins at high levels of ethanol to the presence of bound peptides to the extract matrix that could not be precipitated as we further decreased the solvent polarity.

In order to have a better insight to the surface-active properties of our extracts, we checked the Pearson correlation (r) between total saponins and total proteins contents and interfacial tension. Overall, we found a strong correlation between saponins content and interfacial tension at both MCT oil and soybean oil interfaces ($r = -0.960$ and $r = -0.985$ respectively). However, only a weak relationship was found between proteins content and interfacial tension ($-0.061 \leq r \leq 0.171$). These results indicated that saponins are proposedly contributing to the interfacial properties of the present extract while proteins are less likely participating. In future studies, it would be useful however to carry out a detailed chemical analysis and fractionation study in order to properly understand the interfacial properties of the present extracts.

3.3. Effects of processing conditions on emulsions formation

3.3.1. Effect of extract concentration and homogenization conditions

Following the measurements of interfacial tension, we proceeded to prepare O/W emulsions using 50% (v/v) ethanolic extract as emulsifier. Initially, we evaluated the effect of extract concentration on the Sauter mean diameter ($d_{3,2}$) of 10% soybean O/W emulsions prepared using a high-pressure homogenizer (100 MPa, 4 passes). At low emulsifier concentration (0.1% (w/w)), poor emulsions were obtained with large droplets and signs of instability (oiling-off) in the case of argan extract-based samples (Fig. 3). This type of behavior is likely to be attributed to a limitation in the

emulsifier concentration, assuming that the disruptive forces were sufficiently enough inside of the homogenization chamber (Jafari, Assadpoor, He, & Bhandari, 2008; D. J. McClements, 2016). As we increased the concentration of extract, stable emulsions with narrow droplet sizes were obtained, comparable to those obtained by Tween 20. The smallest droplets ($d_{3,2}$ < 130 nm) were obtained for both emulsifiers at concentrations above 0.5% (w/w). These results confirm the presence of surface-active compounds in the present extract, capable of quickly and efficiently adsorbing at the oil/water interface.

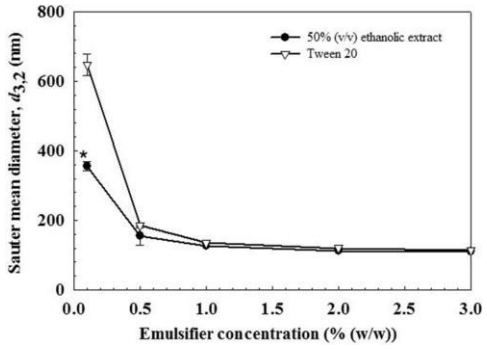

Fig. 3 Effect of emulsifier concentration on the Sauter mean diameter ($d_{3,2}$) of 10% (w/w) soybean O/W emulsions prepared using a high-pressure homogenizer (100 MPa, 4 passes). *A small layer of oil was observed on the top of this sample.

In general, the ability of an emulsifier to produce stable emulsions with narrow droplet diameter depends on many factors such as: (i) its ability to reduce the interfacial tension, (ii) the speed at which it adsorbs on the interface and (iii) its effectiveness to generate repulsive forces during homogenization (Baret, Kleinschmidt, El Harrak, & Griffiths, 2009). Saponins are usually considered as efficient emulsifiers that are capable of adsorbing rapidly on the produced oil droplets during homogenization, due to their relatively small molecular weight and amphiphilic

properties (Yang, Leser, Sher, & McClements, 2013). In addition, the presence of carboxylic acids in their chemical structure, tends to generate a negatively-charged shield around newly synthetized droplets thus limiting their coalescence (Oleszek & Hamed, 2010; Ozturk & McClements, 2016).

In order to further investigate the effectiveness of our extract at producing nanoemulsions with better physical properties, we evaluated the effect of homogenization conditions on the Sauter mean diameter of the prepared emulsions (2% (w/w) emulsifier). Overall, we increased the homogenization pressure and number of passes through the homogenization module so that reducing the Sauter mean diameter, until it reached a fairly constant value ($d_{3,2} \sim 110$ nm) at around 100 MPa and 4 passes (Fig. S1a and Fig. S1b). These observations were in accordance with previous studies that indicated a reduction in droplet size with increasing the intensity or duration of disruptive forces generated by the homogenizer (Bai & McClements, 2016; Qian & McClements, 2011). In addition, the particle size distributions at these homogenization conditions were monomodal with an average span value of 1.35 (Fig. S1c). These results indicated the effectiveness of this extract at producing nanoemulsions at practical operating conditions and with comparable attributes to those obtained when using synthetic emulsifiers such as Tween 20.

3.3.2. Effect of oil mass fraction and oil type

Another important parameter in product engineering of emulsions is the composition of the dispersed phase (Schubert, Ax, & Behrend, 2003). In this section, we evaluated the effect of oil mass fraction and oil type on emulsions formation. Ideally, our objective was to produce stable emulsions using a broad range of commercially applicable oils and at low 'extract-to-oil' ratios. Samples were evaluated for particle size and visual appearance changes after 1 and 10 days of storage at 25 °C.

As shown in Fig. 4a, the smallest $d_{3,2}$ of 107 nm was achieved at fixed homogenization conditions (100 MPa for 4 passes) when 5% (w/w) soybean oil was used as the dispersed phase. Generally, when the entire size distribution of an emulsion is below 80 nm, it becomes optically transparent with better physical attributes than conventional nanoemulsions (Tadros, Izquierdo, Esquena, & Solans, 2004). However, this does not only depend on the emulsifier characteristics or homogenization conditions, but also on the oil phase properties. The nanoemulsions made with relatively high viscous oils such as soybean oil usually present larger droplets and wider size distributions than those prepared with low viscosity oils such as hexadecane (Wooster, Golding, & Sanguansri, 2008).

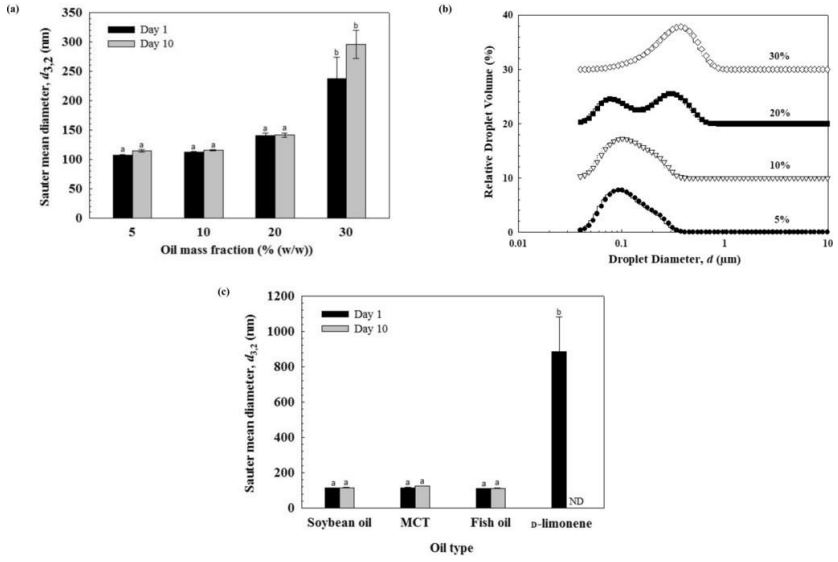

Fig. 4 Effect of oil mass fraction on (a) the Sauter mean diameter ($d_{3,2}$) and (b) droplet size distributions of soybean O/W emulsions containing 2% (w/w) of 50% (v/v) ethanolic extract. (c) Effect of oil type on the $d_{3,2}$ of 10% (w/w) O/W emulsions containing 2% (w/w) of 50% (v/v) ethanolic extract. ND refers to not detected.

The droplet sizes were increased, showing different droplet size distributions, when the percentage of oil was increased in the dispersed phase of emulsions (Fig. 4b). At 10% (w/w) oil fraction, the emulsions presented monomodal distributions with an average droplet diameter of 112 nm. At 20% (w/w) oil, the emulsions became bimodal with larger droplet size ($d_{3,2}$ = 140 nm) but consistent stability over the entire period of storage. Lastly, at 30% (w/w), the emulsions started to present some signs of instability, most likely due to: (i) insufficient presence of emulsifier molecules to cover all newly synthetized droplets, (ii) enhancement of the rate of coalescence and/or (iii) increasing of the viscosity of the system thus suppressing oil droplets breakup during homogenization (Floury, Desrumaux, & Lardières, 2000; D. J. McClements, 2016).

In practical applications, emulsions can be prepared using a variety of different oils. As discussed earlier, variation of the type of the oil strongly affects the properties of emulsions thus putting fundamental challenges in the application of emulsifiers (D. J. McClements, 2016). Therefore, it is important to evaluate the effect of oil type on emulsions formation and stability when using a new emulsifier. Overall, all emulsions presented small droplet sizes ($d_{3,2}$ < 115 nm) and good physical stability, except for those prepared using D-limonene (Fig. 4c). This is due to the fact that essential oils are usually susceptible to Ostwald ripening because of their relatively high solubility in water (Rao & McClements, 2012). Furthermore, they tend to easily oxidize resulting in the formation of off-flavors. Poor-oxidative stability is also problematic when using fish oil for example. In future studies, it would be therefore interesting to evaluate the chemical stability of emulsions prepared using the present extract (50% (v/v) ethanol).

3.4. Effect of environmental stresses on emulsions stability

In order to assess their commercial applications, prepared emulsions (2% (w/w) emulsifier) were subjected to various environmental stresses that might be encountered in practice. These analyses were also informative about the potential mechanism of action of our extract (50% (v/v) ethanol).

3.4.1. Effect of pH

The effect of pH on emulsions stability was analyzed by means of droplet size changes after 24 h of storage at 25 °C. As shown in Fig. 5a, the emulsions prepared using Tween 20 were stable over the entire pH range (1-10) with no evidence of droplet growth or visible instability such as creaming or oiling-off. The emulsions prepared using 50% (v/v) ethanolic extract however, started to present some signs of instability as we reduced the pH. At pH 4 and pH 3, they increased in $d_{3,2}$ (143 and 152 respectively) despite that no visible aggregation have been observed. At pH 2 and pH 1, the emulsions were highly unstable to creaming (data not shown) indicating that droplet flocculation occurred.

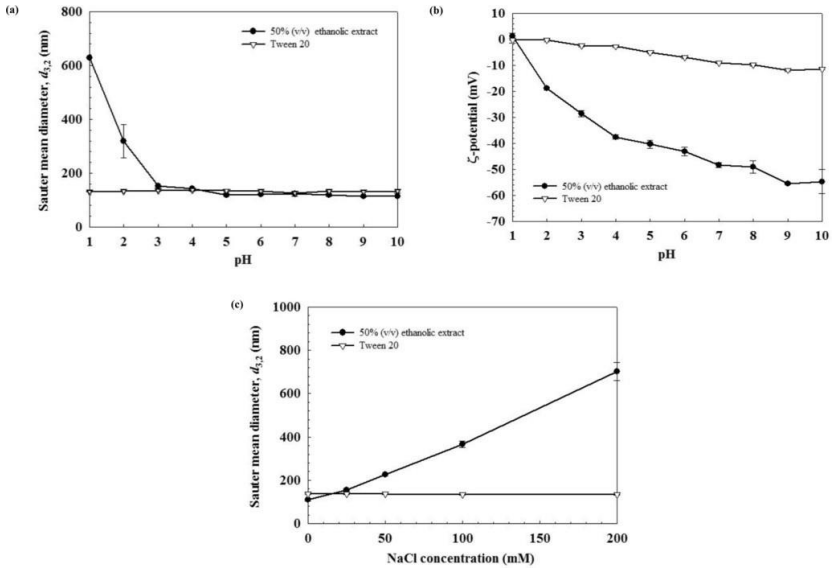

Fig. 5 Effect of pH on (a) the Sauter mean diameter ($d_{3,2}$) and (b) ς-potential of 10% (w/w) soybean O/W emulsions containing 2% (w/w) of Tween 20 or 50% (v/v) ethanolic extract. (c)

Effect of ionic strength on the $d_{3,2}$ of 10% (w/w) soybean O/W emulsions containing 2% (w/w) of Tween 20 or 50% (v/v) ethanolic extract.

In a tentative to understand this behavior, we measured the ζ-potential of the corresponding emulsions (Fig. 5b). Overall, the ζ-potential of emulsions prepared using Tween 20 was less affected by pH due to fact that this is a non-ionic emulsifier with no ionizable groups in its chemical structure. However, we may explain the slight reduction in droplet charge in these emulsions as we increased the pH, with the preferential adsorption of hydroxyl ions (OH^-) on the droplets surface (D. J. McClements, 2016). The emulsions prepared using 50% (v/v) ethanolic extract however, presented a highly negative droplet charge at pH 10 (-54.7 mV) that was gradually neutralized as we reduced the pH, until it reached a fairly positive value at pH 1 (+1.126 mV). Regarding this, we assumed that the main reason of good stability of these emulsions at neutral pH is because of the strong electrostatic repulsions between the highly negatively charged droplets.

As we discussed earlier, the main stabilization mechanism of saponins-based emulsifiers is related of the presence of glucuronic acids in their chemical structure (Yang, Leser, Sher, & McClements, 2013). This latter has a pKa of around 3.25 and consequently tends to be highly negatively charged above this value (Ozturk & McClements, 2016). However, at pH 3, the droplets still had a relatively high negative charge on their surface (-28.5 mV) which indicates that saponins might not be the only surface-active species in this extract.

3.4.2. Effect of ionic strength

The previous observations from section 3.4.1 indicate that electrostatic repulsions plays an important role in the stability of emulsions prepared using 50% (v/v) ethanolic extract. In order to

investigate the contribution of this particular attribute to the overall mechanism of action of this extract and consequently its potential applications, we evaluated the effect of ionic strength on the Sauter mean diameter of the prepared emulsions, after 24 h of storage at 25 °C. As expected, emulsions stabilized by Tween 20 exhibited no evidence of droplet size increment across the entire range of salt levels studied (Fig. 5c). On the other hand, emulsions prepared using 50% (v/v) ethanolic extract were very sensitive to salt addition, displaying an appreciable increase in the droplet size at even low concentrations of NaCl (≥ 25 mM). These results indicated again that Tween 20 is a non-ionic emulsifier, while emulsions containing 50% (v/v) ethanolic extract are exclusively stabilized because of the electrostatic repulsions between oil droplets.

3.5. Effect of long-term storage

In practice, emulsion-based products are supposed to remain stable throughout their entire shelf-life. For this reason, we evaluated the long-term storage stability of emulsions prepared using Tween 20 or 50% (v/v) ethanolic extract, at different incubation temperatures (5, 25 and 45 °C). Overall, no changes in visual appearance were observed throughout the entire period of storage. However, a small increase in droplet size (Table 1) was obtained in the argan extract-based emulsions, when stored at 45 °C ($d_{3,2}$ = 146 nm). These results indicated that both emulsifiers were efficient at preventing droplets from coalescing by using different mechanisms of action. Tween 20-coated droplets are mainly stabilized against aggregation because of their extended polymeric head groups (Teo, Goh, Wen, Oey, Ko, Kwak, et al., 2016). Fifty percent (v/v) ethanolic extract however, contains surface-active species that strongly ionize at neutral pH thus providing sufficient electrostatic repulsions between droplets.

Table 1: Particle size changes ($d_{3,2}$) of emulsions containing 2% (w/w) of Tween 20 or 50% (v/v) ethanolic extract and prepared at standardized homogenization conditions (100 MPa, 4 passes).

	50% (v/v) ethanolic extract (nm)			Tween 20 (nm)		
	Day 1	Day 30	Day 60	Day 1	Day 30	Day 60
5 °C	110	104	118	114	120	119
25 °C	114	113	118	113	121	115
45 °C	113	132	146	114	121	114

4. Conclusions

In this study, we evaluated the interfacial, emulsion forming and emulsion stabilizing properties of a natural extract from argan oil press-cake. We started the experiments by comparing the interfacial tensions of different extracts prepared from this material. Following this, we selected 50% (v/v) ethanolic extract for emulsions preparation. Our results clearly showed that this extract was capable of producing nanoemulsions with narrow droplet size ($d_{3,2}$) and good physical stability comparable to those obtained when using Tween 20. The main stabilization mechanism is believed to be exclusively electrostatic, likely due to the presence of surface-active compounds with ionizable groups such as saponins. On the other hand, the prepared emulsions were highly unstable to salt addition (≥25 mM) and to extreme acidic pH (<3) which may limit their application in the future. Furthermore, for potential application in the food and beverage industries it would be important to extend this work by evaluating other parameters such as the taste profile, the potential toxicity, the cost and the reliability of supply.

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Conflict of interest

The authors declare that there are no conflict of interest relating to the experimental results presented herein.

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