The Emulsifying Performance of Mildly Derived Extracts from Argan By-products:

Towards a Sustainable Production of Natural Emulsifiers

A Dissertation Submitted to the
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Abstract

Argan oil extraction industry generates every year large amounts of by-products that are not efficiently valorized. On average, 1 ha of argan trees can produce around 300 kg of dried fruits, in the form of 20 kg of kernels and only 8 kg of oil. This does not only result in the loss of potentially high value-added compounds from these materials but also impacts the local environment, through overexploitation of argan trees for fruit harvesting and oil extraction. Regarding this, a systematic study of the secondary metabolites of argan tree was initiated since the early ’90s. The objective is to identify new bioactive compounds that can increase the tree’s economic and industrial values. The results of this survey allowed to characterize, within the different parts of the argan tree, a wide variety of functional compounds, with some of them already evaluated for different biological activities.

In the introductory section, we presented an overview of the different valorization opportunities of argan by-products in cosmetic and pharmaceutical applications. We also pointed out the great potential of obtaining natural emulsifiers from agro-industrial by-products. Our aim was to suggest the use of argan by-products as a source of natural emulsifiers. In our view, the rich surface-active composition of these materials (e.g. proteins, saponins), as well as their significant generation quantities, can create potential applications as natural emulsifiers.

In chapter 2, we evaluated the surface-active and emulsifying properties of saponin-rich extracts from argan oil press-cake. Our aim was to produce model oil-in-water (O/W) nanoemulsions using these extracts as sole emulsifiers. Various extracts were initially 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 medium chain triglycerides (MCT) oil and soybean oil interfaces. This extract was also effective at producing stable nanoemulsions using

different oils such as soybean oil, MCT oil and fish oil, and with similar properties to those obtained by conventional emulsifiers such as Tween 20.

In chapter 3, we evaluated the physical stability and biological fate of emulsions prepared using argan extract. The emulsions were very sensitive to salt addition (≥25 mM) and to extreme 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. The emulsions were also very sensitive to gastric conditions, particularly when pepsin was added to the digestion system, which highlights the contribution of proteins to the surface-active and emulsifying properties of this extract.

In chapter 4, we evaluated the emulsifying performance of argan extract in microchannel emulsification (MCE). Our aim was to produce stable monodisperse O/W emulsions using this extract a sole emulsifier. The complex composition of this extract imparted its emulsifying efficiency, by creating a hydrophobic, or slightly hydrophilic, layer on the MC array plate surface. This resulted in unsuccessful emulsification using short MCs but did not affect the emulsification efficiency in longer ones. Using these longer channels, we could produce stable monodisperse O/W emulsions, with similar droplet size and droplet size distribution to those obtained by Tween 80, and for up to 10 h of continuous emulsification.

In chapter 5, we compared the surface-active and emulsifying properties of a crude extract from licorice root and purified saponins from the same origin. Our aim was to examine the contribution of specific compounds to the emulsifying performance of multicomponent plant extracts. As expected, the non-purified extract was more surface-active and effective at producing small droplet size emulsions, in comparison to purified saponins. The emulsions were superiorly stable at low pH and high salt concentrations but less stable at elevated temperatures, suggesting again the

contribution of proteins to the observed results. Evidences presented in this chapter indicated that non-purified surface-active extracts, such as argan and licorice root extracts, can provide superior emulsifying ability in comparison to purified emulsifiers. This is due to the contribution of multiple compounds to the overall surface-active and emulsifying properties, and most likely the formation of biogenic complexes between these compounds at the oil/water interface, thus leading to improved droplet coverage and stability.

In the final section, we suggested innovative scenarios for the valorization of argan by-products. We believe that sequential extraction methods should be adopted in the future to simultaneously obtain various bioactive and dietary compounds from these materials. Suitable storage conditions, innovative harvesting methods and adjusted oil production chains should be also considered in order to preserve specific natural compounds in the different parts of argan fruit, without affecting the productivity of the tree. Finally, for successful application in the food and beverages industry, other parameters such as bitterness, the potential toxicity, the cost and the reliability of supply are important.

Contents

Introduction

Motivation

Over the last decade, the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA) have funded more than 130 international research projects (SATREPS) all around the world. The objective is to address global issues in recipient countries and contribute to their sustainable development, through science and technology, by producing a wide variety of research outcomes and ensuring their utilization in society. On September 17th, 2016, scientists from Japan, Morocco and Tunisia deliberated the kick-off symposium of a joint SATREPS project between the three countries. The project is entitled *"Valorization of Bio-resources in Arid and Semi-Arid Land based on Scientific Evidence"* and aims to the development of food, medicinal and cosmetic products from Moroccan and Tunisian bioresources, and their authentication for the creation a new industry [1].

Multiple plant resources have been initially targeted, including argan, olive and prickly pear. In Morocco, these plants are generally confronted with harm and stressful environmental conditions, which promotes the synthesis and accumulation of active metabolites [2]. Argan oil, for example, is known to contain a rich and particular composition of bioactive compounds, including polyphenols, tocopherols, sterols and triterpenes [3]. These compounds are naturally bioactive and are generally associated with multiple curative and cosmetic properties. Interestingly, these compounds are also present (often in higher concentration) in the by-products obtained after oil extraction [4]. If the functionality of these compounds can be shown and if they can find innovative applications in food, cosmetic or pharmaceutical products, they can promote a sustainable industry,

within a win-win situation for both the local environment and the benefiting industrials. This work underscores this exact motivation, with special focus on argan oil extraction by-products.

Valorization opportunities of argan by-products

Argan (*Argania spinosa*) is an endemic plant species of Morocco. It thrives naturally in the southwestern parts of the country over an estimated area of 830 000 ha, thus covering more than 17% of the Moroccan forest [5]. Argan oil is the main product of the argan tree. It is extracted from the fruit kernels and is famous throughout the world for having multiple dietary and cosmetic properties. The detailed procedure of argan oil extraction has been previously reported in several studies and therefore will be only described briefly hereafter. First, the fruits are dried in the sun and the pulp (mesocarp) is manually removed by workers. Next, the hard nuts (endocarp) are crushed with stones to afford 1 to 3 kernels per fruit. Finally, the kernels are pressed, leaving behind a brownish-yellowish dough, that is subsequently processed or discarded, according to the extraction method [3]. Despite the continuous efforts to optimize this process, the yields of extraction still generally range from 2-3.5 kg of oil/100 kg of dried fruits [6]. This means that more than 95% of the initial material is converted into low-value by-products that are not efficiently valorized (Appendix: Table 1).

In this section, we briefly reviewed the different valorization opportunities of argan by-products in food, cosmetic and pharmaceutical applications. The chemical profile, biological properties and real dermo-cosmetic application examples of argan pulp, press-cake and nutshell are provided in appendices (Appendix: Tables 2, 3, 4 and 5). The review highlights several bioactive compounds from argan by-products. Many of these compounds have been included previously in cosmetic or dermopharmaceutical formulations, under the label of skin or hair care agents from the argan tree. Interestingly, only limited information is available about their application in food products. In our

view, the rich chemical composition of these by-products, as well as their significant production quantities, can open potential applications, not only in medicinal and cosmetic products but also in foods and/or beverages.

Natural emulsifiers from agro-industrial by-products

Emulsifiers are amphiphilic molecules or particles, capable of adsorbing at the hydrophile-hydrophobe interface, thus reducing the tension between immiscible liquids. Due to their surface-activity and versatile properties, emulsifiers have various applications over a broad range of industries. Particularly, they are used as stabilizers and/or encapsulants in food and/or beverages in which they provide multiple properties such as good dispersibility, prolonged stability and improved bioavailability of other ingredients [7]. For many years, the emulsifiers industry has established various processes for the production of emulsifying compounds, with proven properties and competitive prices. Consequently, many of the emulsifiers used nowadays still derive from the same chemical and/or enzymatic reactions that were primarily designed in the past for mass and economic production of these substances [8]. The current trend however towards natural and sustainable products have necessitated manufacturers to find new natural alternatives to synthetic emulsifiers [9]. As a result, intensive research has been done to extract, identify and characterize various natural emulsifying compounds from different origins.

In this section, we briefly reviewed the different emulsifying compounds obtained from agro-industrial by-products. Saponins, protein and polysaccharides have been successfully obtained previously and evaluated for their emulsifying/stabilizing properties. Agro-industrial by-products have been also used as substrates for the production of biosurfactants by microorganisms. Nevertheless, for potential use in the food and beverage industries, we believe that other parameters, such as the preparation cost and environmental impact must be also considered. In our

view, the use of crude plant emulsifying extracts, obtained via simple extraction/separation steps, can be of additional value to the final product.

Research objective and thesis outline

In the previous sections, we presented a brief overview of the different valorization opportunities of argan by-products. We also pointed out the great potential of obtaining natural emulsifiers from agro-industrial by-products. Our aim was to suggest the use of argan by-products as a source of natural emulsifiers. In our view, the rich surface-active composition of these materials (e.g. proteins, saponins), as well as their significant generation quantities, can create potential applications as natural emulsifiers.

In a systematic procedure, we started the study by evaluating the surface-active and emulsifying properties of various saponins-rich extracts from argan press-cake (Chapter 2). Our objective was to keep the extraction procedure as simple as possible, without further purification or fractionation of the obtained composition. Next, we evaluated the stability and biological fate of prepared emulsions and provided initial insights into their emulsification mechanism (Chapter 2). Until this point, however, it was not clear how the mutual presence of multiple surface-active compounds, such as proteins and saponins, would affect the emulsifying properties of the entire composition. We assessed, therefore, the emulsifying behavior in microchannel emulsification, which could provide additional insights about the overall emulsification properties (Chapter 4). In chapter 5, we were more interested in confirming our findings, by comparing the surface-active and emulsifying properties of a purified saponin (glycyrrhizin) and non-purified glycyrrhizin-rich extract from licorice root. From this comparative study, we could underline conclusive remarks about the emulsification mechanism of multicomponent compositions, such as argan and licorice root extracts.

Chapter 1

Formulation characteristics of O/W nanoemulsions prepared using a saponin-rich extract from argan oil-press-cake

Abstract

Various aqueous-ethanolic extracts were prepared from argan press-cake 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). Our results indicate that the non-purified surface-active extracts from argan oil press-cake can have attractive applications as new natural emulsifiers. Nevertheless, for potential use in the food and beverage industries, other parameters such as the stability characteristics and the biological fate of prepared emulsions must be also evaluated.

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Introduction

Emulsions are one of the most important delivery systems in food, cosmetic and pharmaceutical industries. 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 [7]. 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 [10]. 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 [9].

Recently, there has been increased 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. Among these, proteins (e.g. caseins, whey proteins), polysaccharides (e.g. starch, pectin), phospholipids (e.g. lecithin) and saponins are relevant [11, 12, 13]. More recently, a food-grade emulsifier made from the bark of *Quillaja saponaria* tree had been successfully used to prepare O/W nanoemulsions with narrow droplet size and with improved stability over a wide range of environmental stresses [14]. 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 [15]. 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 [16]. 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 [9]. It was therefore suggested to identify other commercially viable sources of such natural emulsifiers.

In this chapter, we evaluated the surface-active and emulsifying properties of saponin-rich extracts from argan oil press-cake. Our aim is to produce stable model O/W emulsions using this extract as sole emulsifier.

Materials and methods

Materials

Cold-pressed argan kernels cake was provided by an oil-producing cooperative in the region of Essaouira, Morocco and stored at 4 °C until use. Ethanol, oleanolic acid standard (97%), bovine serum albumin (BSA), Bradford reagent, refined soybean oil, D-limonene, polyoxyethylene (20) sorbitan monolaurate (Tween 20), sodium azide, 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). Whatman paper was obtained from GE Healthcare Life Sciences (Chicago, Illinois, USA). Disposable syringe filters (0.22 μm pore size, Nylon) were obtained from Advantech 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.

Preparation of argan extracts

The press-cake was finely ground (< 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 bleaching 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 concentrations.

Chemical analysis of argan extracts

Total saponins content was spectrophotometrically determined (V-570, JASCO Co., Hachioji, Japan) according to the method of Xiang et *al*. [17], 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 down 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 a 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 Bradford's method [18]. 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 the extract was expressed as BSA equivalent using a BSA standard curve (0-1.4 mg mL^{-1}).

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.

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 a different number of passes (1-10).

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.

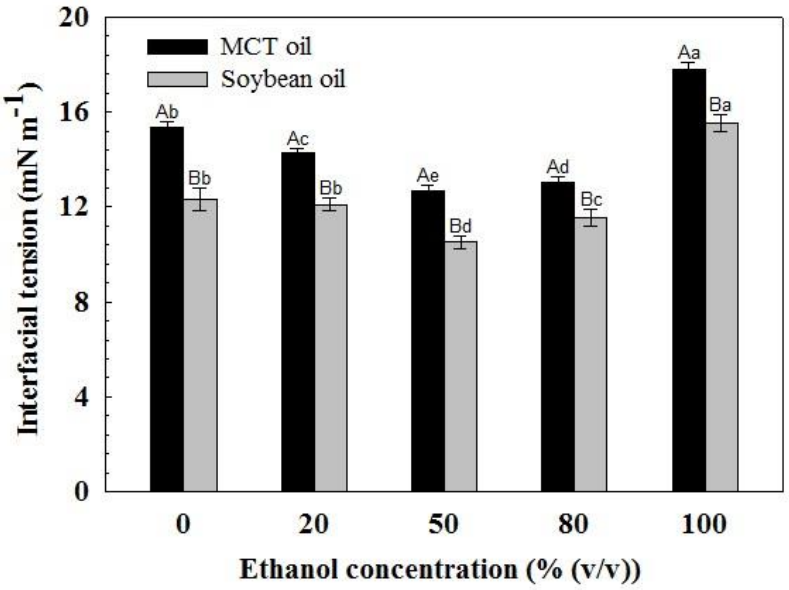
Results and discussion

Interfacial properties of aqueous-ethanolic extracts

Argan oil press-cake is known to contain large amounts of saponins [19]. These compounds are also known to have surface-active properties, potentially acting as emulsifiers [20]. Since more than one method for argan saponins extraction are reported [21], 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 (Figure 1). 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. 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.

Interfacial tension of surfactants plays an important role in determining their ability to form and stabilize emulsions [22]. Usually, the lower is the interfacial tension, the greater is the emulsifying property [23]. For this reason, we selected 50% (v/v) ethanolic extract for 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 (Figure 1). Using the method described previously by Benincasa et *al*. [24], 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.

(a)



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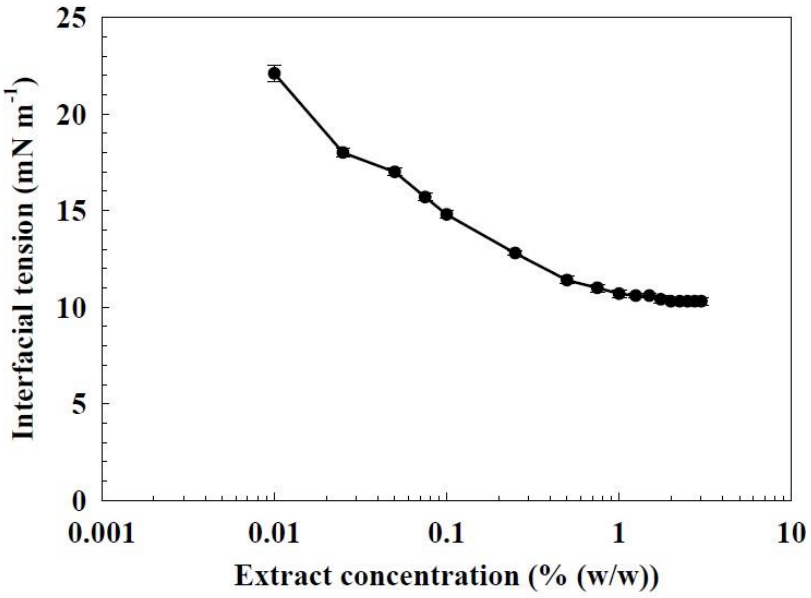


Figure 1. Effect of the extraction solvent on the interfacial tension of newly created interfaces. (a) Effect of extract type (1% (w/w)) on the interfacial tension at the oil/water interface. (b) Interfacial tension as a function of 50% (v/v) ethanolic extract concentration at the soybean oil/water interface. 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.

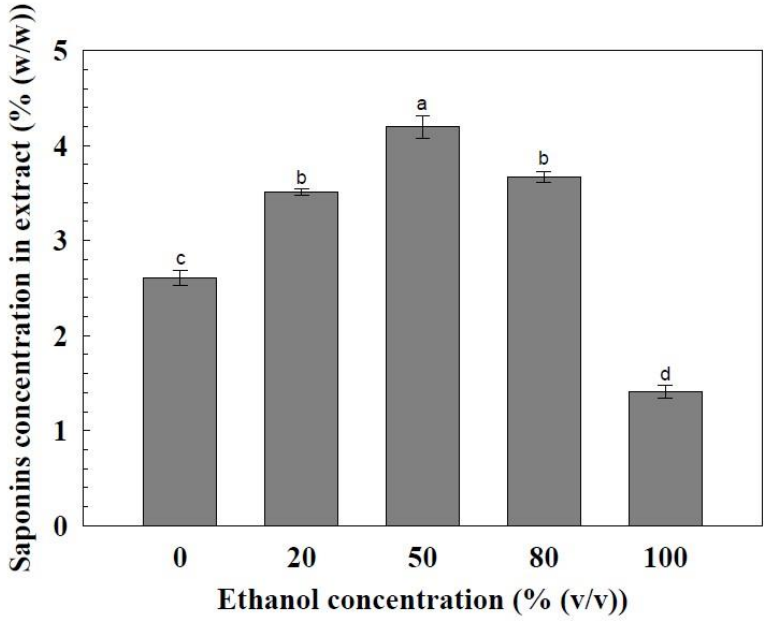
Saponins and proteins contents in aqueous-ethanolic extracts

Saponins and proteins are known to have surface-active properties because of the presence of hydrophilic and lipophilic moieties in their chemical structures [12]. Therefore, we specifically targeted these compounds in the prepared extracts in order to investigate the contribution of specific ingredients to the obtained interfacial properties. Overall, total saponins and total proteins contents were significantly affected by the 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 (Figure 2). Similar results were previously obtained by Henry et *al*. [25]. 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 [19]. However, in almost all former studies, extraction was only performed using high concentrations of ethanol or methanol [21, 26]. The results depicted in Figure 2 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 et *al*. [27], 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.

For proteins analysis, it is generally known that ethanol causes proteins precipitation due to structural and conformational modifications [28]. 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)) (Figure 2). 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

(a)



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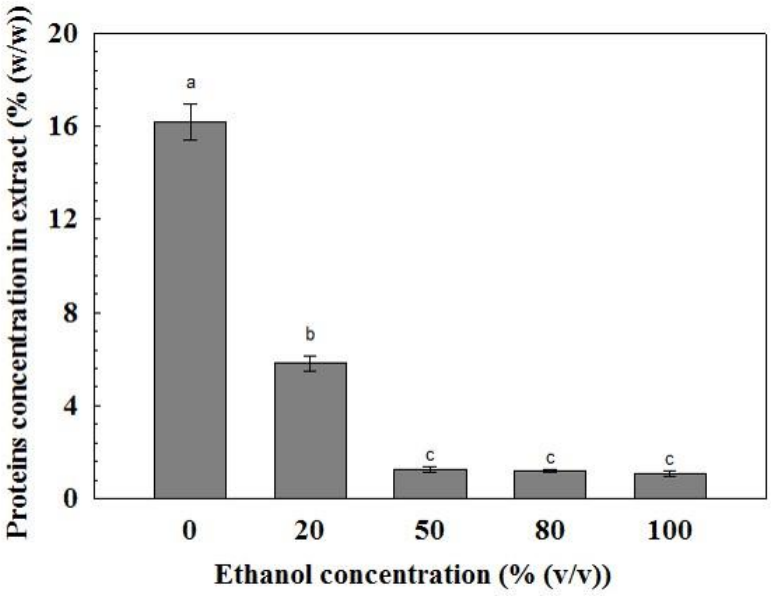


Figure 2. Effect of ethanol concentration on (a) total saponins and (b) total proteins contents in argan extracts.

carbohydrate contents. However, no previous studies have analyzed the proteins profile 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.

Effect of extract concentration on emulsion formation

Following the measurements of interfacial tension, we proceeded to prepare O/W emulsions using 50% (v/v) ethanolic extract as an 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 (Figure 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 [29]. As we increased the concentration of extract, stable emulsions with narrow droplet sizes were obtained, comparable to those obtained by Tween

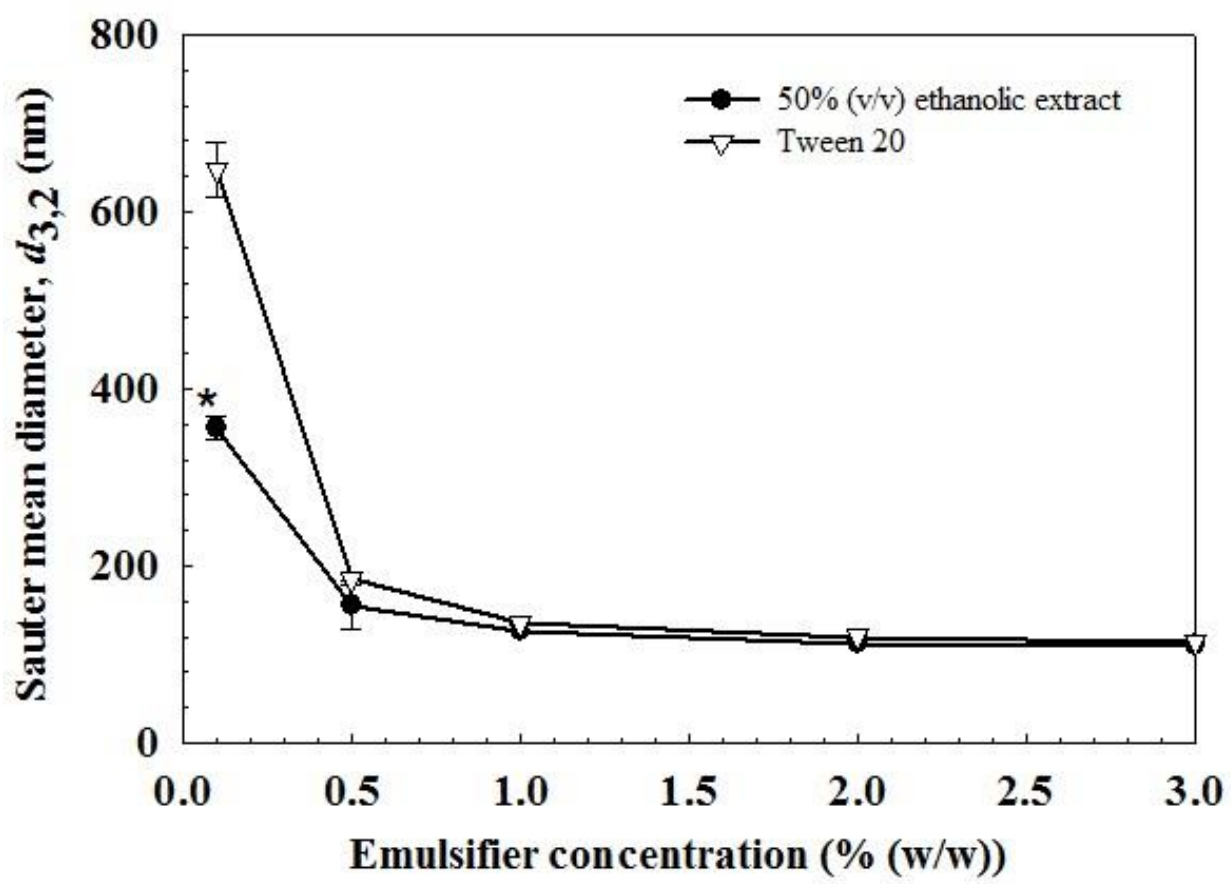


Figure 3. Effect of emulsifier concentration on the Sauter mean diameter ($d_{3,2}$) of 10% (w/w) soybean O/W emulsions prepared using a high-pressure homogenizer (100 MPa, 4 passes). *A small layer of oil was observed on the top of this sample.

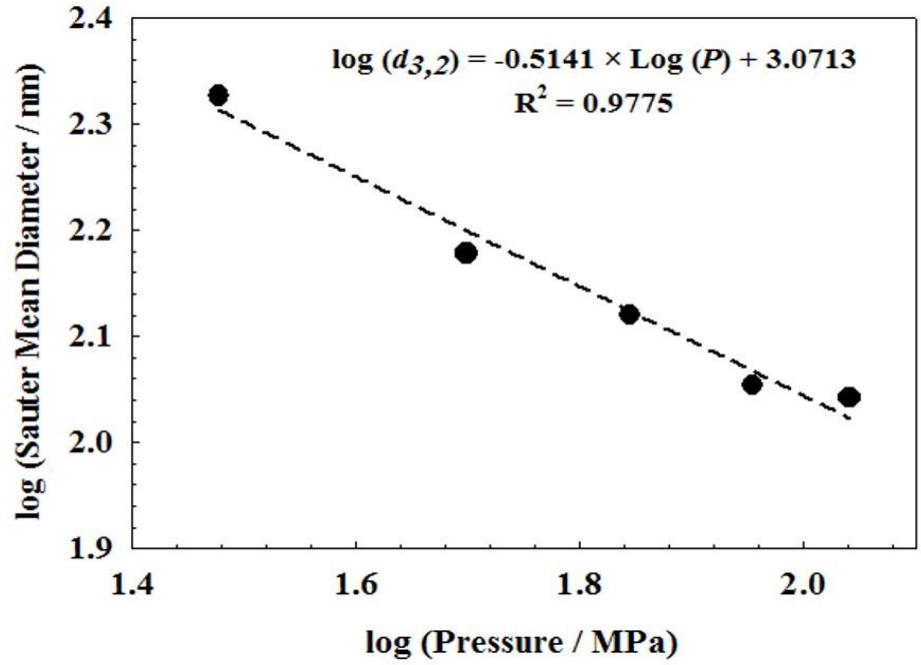
20. The smallest droplets ($d_{3,2}$ < 130 nm) were obtained for both emulsifiers at concentrations above 0.5% (w/w). These results confirm the presence of surface-active compounds in the present extract, capable of quickly and efficiently adsorbing at the oil/water interface.

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 [30]. 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 [14]. In addition, the presence of carboxylic acids in their chemical structure tends to generate a negatively-charged shield around newly generated droplets thus limiting their coalescence [9, 20].

Effect of homogenization conditions on emulsion formation

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 the number of passes through the homogenization module so that reducing the Sauter mean diameter, until it reached a fairly constant value ($d_{3,2}$ ~ 110 nm) at around 100 MPa and 4 passes (Figure 4). 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 [31]. In addition, the particle size distributions at these homogenization conditions were monomodal with an average span value of 1.35. These results indicated the effectiveness of this extract at producing nanoemulsions at practical operating conditions and with comparable attributes to those obtained when using Tween 20.

(a)



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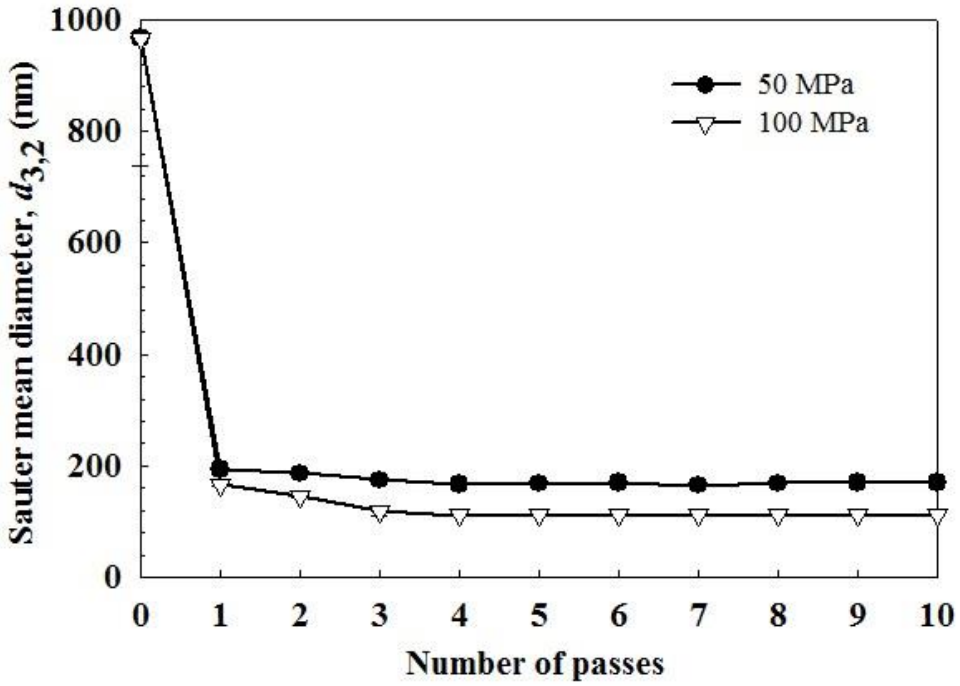


Figure 4. Effect of (a) homogenization pressure and (b) number of passes through the high-pressure homogenizer (100 MPa) on the Sauter mean diameter ($d_{3,2}$) of 10% (w/w) soybean O/W emulsions containing 2% (w/w) of 50% (v/v) ethanolic extract.

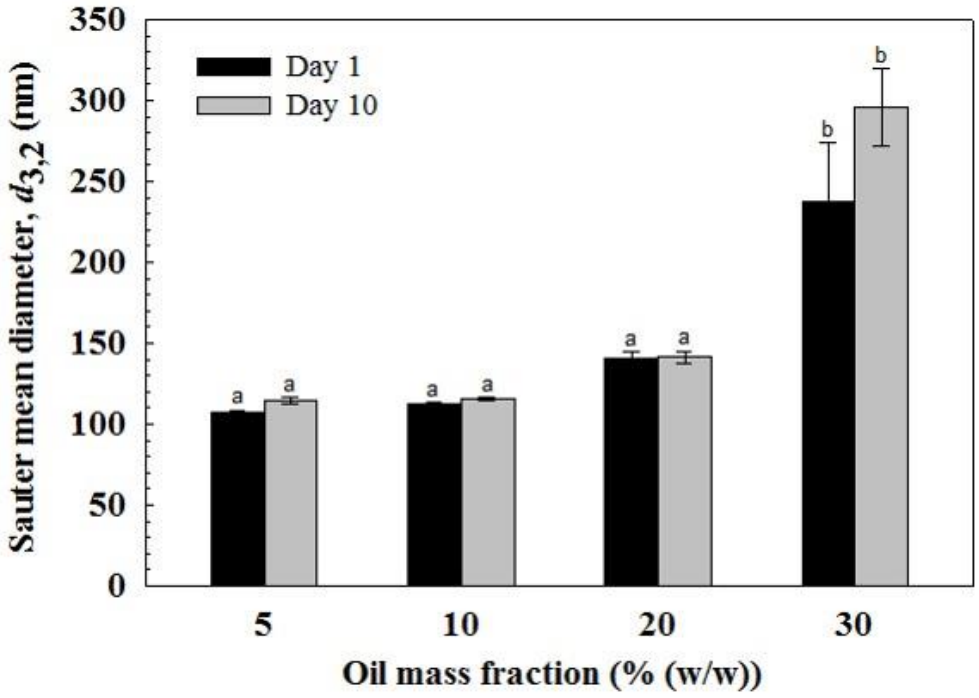
Effect of oil mass fraction and oil type on emulsion formation

Another important parameter in product engineering of emulsions is the composition of the dispersed phase [32]. 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 Figure 5, 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 [32]. 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 [33].

The droplet sizes were increased, showing different droplet size distributions when the percentage of oil was increased in the dispersed phase of emulsions (Figure 5). 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 [34].

(a)



(b)

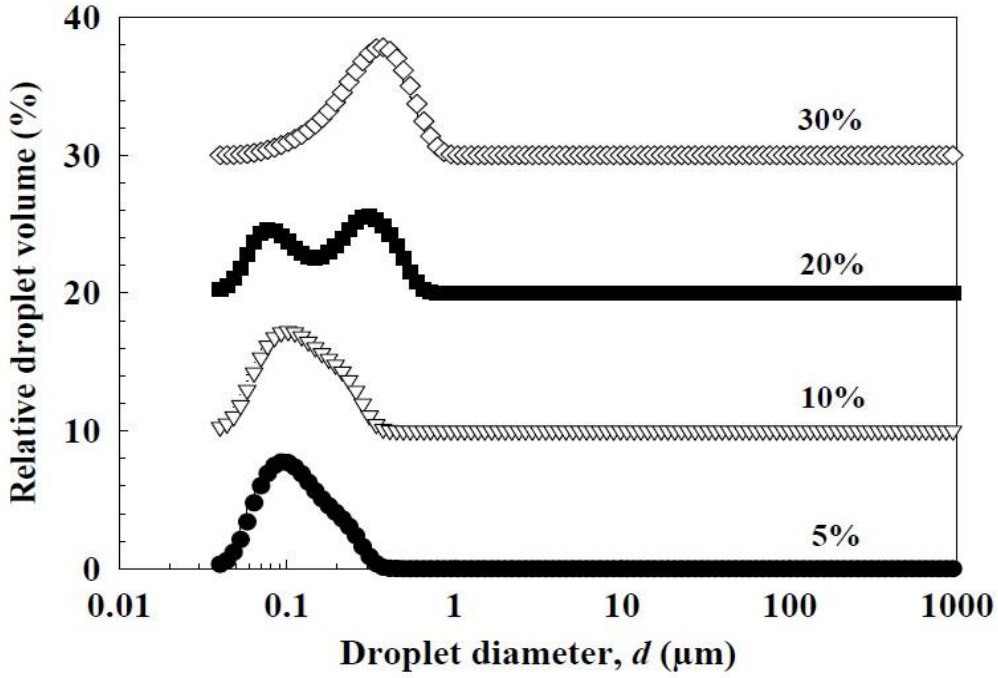


Figure 5. Effect of oil mass fraction on (a) the Sauter mean diameter ($d_{3,2}$) and (b) droplet size distributions of soybean O/W emulsions containing 2% (w/w) of 50% (v/v) ethanolic extract.

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 [7]. 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 (Figure 6). This is due to the fact that essential oils are usually susceptible to Ostwald ripening because of their relatively high solubility in water [35]. 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 interesting to evaluate the chemical stability of emulsions prepared using the present extract (50% (v/v) ethanol).

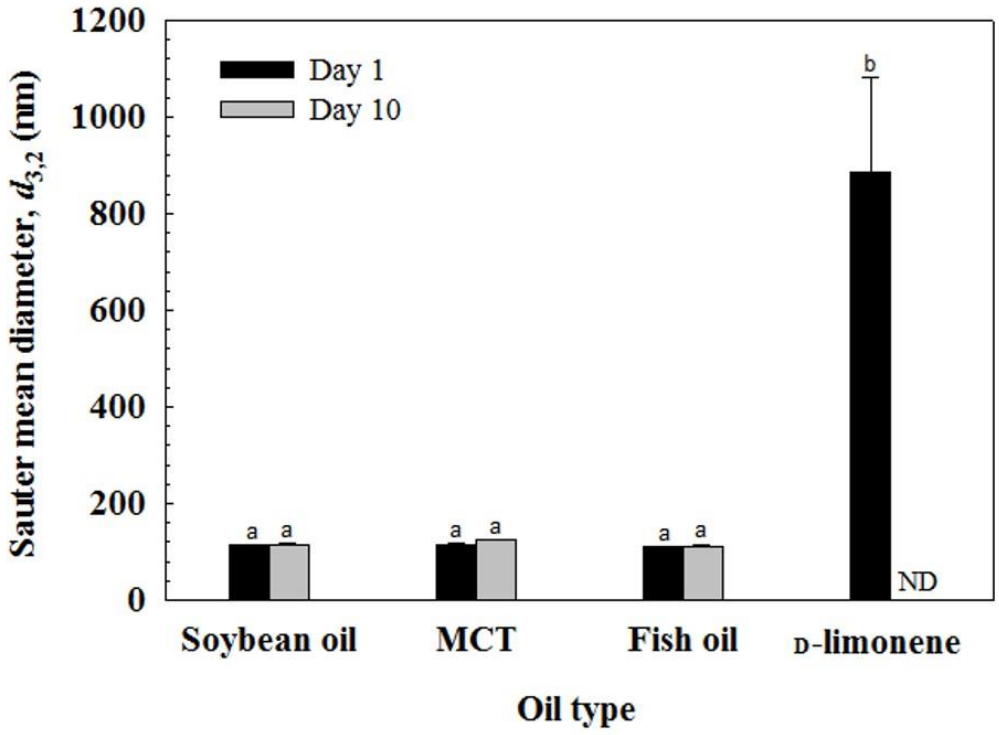


Figure 6. Effect of oil type on the Sauter mean diameter ($d_{3,2}$) of 10% (w/w) O/W emulsions containing 2% (w/w) of 50% (v/v) ethanolic extract. ND: Not detected.

Chapter 2

Stability evaluation and *in-vitro* gastric digestion of O/W emulsions prepared using a surface-active extract from argan oil press-cake

Abstract

Emulsions prepared using argan extract were very sensitive to salt addition (≥25 mM) and to extreme 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. The emulsions were very stable to storage conditions but less resistant to gastric digestion, particularly when pepsin was added during to the digestion system. This highlights the importance of proteins in the stability of these emulsions despite their low concentration in the initial extract (~ 1%, w/w). However, it is not clear how the contribution of multiple surface-active compounds, such as proteins and saponins, would affect the emulsifying properties of the entire composition.

Sections of this chapter are published as:

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Introduction

Emulsions are one of the most applied colloidal systems over a broad range of industries. Products such as paint, salad dressings and face lotions are all essentially emulsions. They are thermodynamically unstable systems of two or more immiscible liquids and therefore tend to eventually break down at certain conditions [7]. According to their application however, emulsions may require different physicochemical properties and preparation characteristics. In beverage emulsions for example, factors such as droplet size and concentration are important [36]. In cosmetic and pharmaceutical emulsions on the hand, other parameters such as droplet size distribution and phase composition are more relevant [37]. In all these cases however, emulsions stability is necessary for perceived quality of the final product. Normally, it is expected that the emulsion remains stable throughout the entire period of storage and resist to the different environmental conditions that may be encountered during its shelf life.

In practice, it is generally difficult to quantitively predict the stability of emulsions-based products by simple evaluation of their preparation characteristics. Nevertheless, it is possible to assess certain changes of their quality by systematic analysis of their composition and theoretically understanding the physicochemical properties of the main ingredients (e.g. emulsifiers) [7]. Polysorbate-based emulsions for example are excepted to show good stability over a broad range of pH [14]. Lecithin are known to be sensitive to acidic pH [13], while protein emulsifiers are more likely to destabilize at pH close to their isoelectric point [38]. In all these cases however, the droplets interface is usually uniform which provides established characteristics to the final product. In the case of mixed emulsifiers systems on the other hand, the interface is much more complexed and variable, thus the need to experimentally monitor the influence of the different environmental stresses on emulsions stability [39].

In general, the understanding of mixed emulsifier systems is still relatively poor, which requires specific design guidelines for rational application. Nevertheless, it has been shown previously that the combination of multiple emulsifiers from different origins could result in improved stability of prepared emulsions. Reichert et *al*. [40] for example found that the utilization of Quillaja saponin-sodium caseinate binary mixtures results in stable O/W emulsions, with properties being tunable according to the mixing ratios of the two emulsifiers. Other findings also include the beneficial effect of mixed emulsifier systems to enhance emulsion stability towards coalescence, flocculation, gravitational separation and Ostwald ripening [41, 42, 43, 44].

The approach of combining purified emulsifiers after many steps of laborious separation however is intriguing. In many cases, these emulsifiers are originally present in the same matrix and do not have to be separated in the first place. The design of less intensive approaches for the application of mixed emulsifier systems is therefore necessary and must eventually lead to the reconsideration of the whole biorefinery practices. An alternative approach for the application of mixed emulsifiers systems suggests the use of crude plant emulsifying extracts, obtained via simple extraction/separation steps for sustainable production of emulsion-based products [45, 46]. These extracts are a native mixture of multiple compounds that can act together to provide specific interfacial properties and stability characteristics to the prepared emulsions [47, 48, 49]. In these extracts however, the composition is much more complex and variable, thus the difficulty to predict emulsions stability. Ralla et *al*. [50] for example found that two different extracts from ginseng root have similar interfacial properties and resembling surface-active composition but display different emulsifying performance when applied at the same conditions. We therefore believe that for a rational application of such complex compositions, it is important to systematically evaluate the effect of the different environmental conditions on emulsions stability.

In this chapter, we evaluated the impact of external stress such as salt and pH on the stability of emulsion prepared using a multicomponent emulsifying extract from argan press-cake. We also evaluated the biological fate of prepared emulsions at gastrointestinal conditions, in the hope to provide additional insights about the emulsions stabilizing mechanisms of this extract in O/W emulsions.

Materials and methods

Materials

Argan kernels cake was provided by an oil-producing cooperative in the region of Essaouira, Morocco. Ethanol, soybean oil, polyoxymethylene (20) sorbitan monolaurate (Tween 20), sodium azide, sodium chloride, sodium hydroxide and hydrochloric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Whatman paper was obtained from GE Healthcare Life Sciences (Chicago, Illinois, USA). Disposable syringe filters (0.22 μm pore size, Nylon) were obtained from Advantech Co., Ltd. (Taipei, Taiwan). Deionized water (18 MΩ cm) was produced by a Milli-Q system (Arium® pro, Göttingen, Germany) and used to prepare all solutions and emulsions in the current study.

Preparation of argan extract

Argan press-cake was finely ground to around 0.5 mm and extracted for 2 hours using distilled water and ethanol (50:50, v/v). After centrifugation (MX-307, Tomy Digital Biology Co., Ltd., Tokyo, Japan) and filtration to remove solid particles, the solvent was then 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 concentrations.

Preparation of O/W nanoemulsions

Emulsions were prepared using 10% (w/w) soybean oil as dispersed phase and 90% (w/w) argan extract solution (in 10 mM phosphate buffer) as continuous phase. First, 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. The coarse emulsion was then passed through a high-pressure homogenizer (NanoVater, NV200, Yoshida Kikai, Nagoya, Japan) at 100 MPa and 4 passes to obtain a fine nanoemulsions.

Effect of pH on emulsions stability

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

Effect of salt on emulsions stability

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

Effect of long-term storage on emulsions stability

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.

In vitro gastrointestinal digestion of O/W emulsions

The prepared emulsions were passed through a two-step simulated gastrointestinal model that consisted of a stomach and intestinal phase. The particle size, microstructure and free fatty acid (FFA) release of the samples were analyzed after each stage.

The simulated gastric fluid (SGF) was prepared by dissolving 2 g of NaCl, 7 mL of HCl (35–37%) and 3.2 g of pepsin in 1 L of Milli-Q water. The samples (20 g) were then added to 20 g of this SGF, and the pH of the mixture was adjusted, for 2 hours under continuous stirring (100 rpm) and at 37 °C, by using a 2.5 M NaOH solution.

Digesta samples (30 g) collected from the stomach phase were added to 4 mL of bile extract solution (46.8 mg mL^{-1} in phosphate buffer, pH 7) and 1 mL of calcium solution containing 110 mg of $CaCl_2$, and then the pH was adjusted to 7 using NaOH solution (1 M). Next, 2.5 mL of freshly prepared pancreatin suspension (24 mg mL^{-1} in phosphate buffer, pH 7) was added to the mixture, and the pH was maintained at 7.0 by adding NaOH solution (0.5 M) to neutralize the FFA released during digestion. The volume of the added NaOH was recorded during 2 h of digestion and was used to calculate the FFA release using the following equation:

$$\%\mathrm{FFA}\,(t) = \frac{V_{\mathrm{NaOH}}(t) \times M_{\mathrm{NaOH}} \times m_{\mathrm{Lipid}}}{2 \times W_{\mathrm{Lipid}}} \times 100,$$

where V_{NaOH} (t) is the volume (L) of NaOH solution titrated at digestion time (min), M_{NaOH} is the molarity of NaOH used (M), m_{Lipid} is the molecular weight of soybean oil (g mol^{-1}), and W_{Lipid} is the total mass (g) of soybean oil initially present in the samples during digestion in the small intestinal phase.

Particle characterization

Particle size measurements were conducted using a laser diffraction particle size analyzer (LS 13320, Beckman Coulter, Brea, USA). Droplet charge was determined using a ζ-potential analyzer (Zetasizer, Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). Microstructure observation were conducted using a confocal scanning laser microscope (TCS SP8; Leica Microsystems GmBH, Wetzlar, Germany).

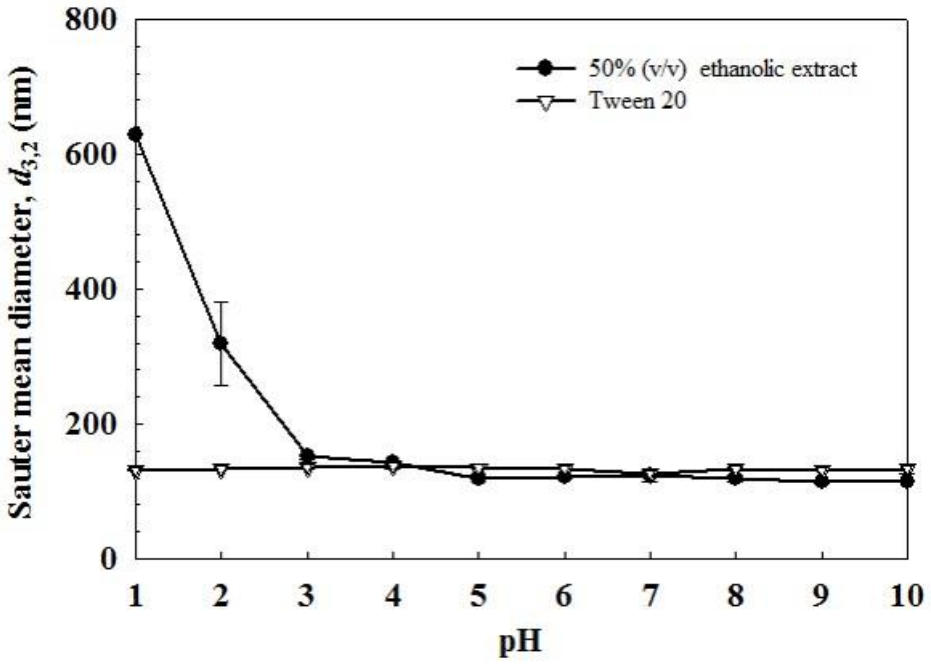
Results and discussion

Effect of pH on emulsions stability

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 Figure 7, 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 on the other hand, 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 nm respectively) despite that no visible aggregation has been observed. At pH 2 and pH 1, the emulsions were highly unstable to creaming (data not shown) indicating that droplet flocculation occurred.

In a tentative to understand this behavior, we measured the ς-potential of the corresponding emulsions (Figure 7). Overall, the ς-potential of emulsions prepared using Tween 20 was less

(a)



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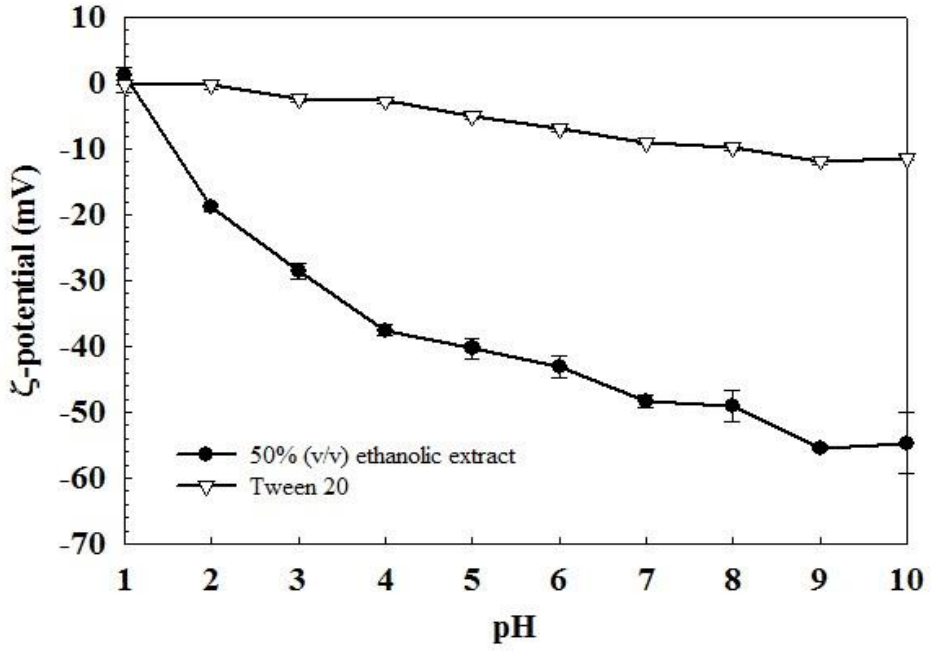


Figure 7. Effect of pH on (a) the Sauter mean diameter ($d_{3,2}$) and (b) ς-potential of 10% (w/w) soybean O/W emulsions containing 2% (w/w) of Tween 20 or 50% (v/v) ethanolic extract.

affected by pH due to the 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 [7]. 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 for 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 to the presence of glucuronic acids in their chemical structure [14]. These latter have a pKa of around 3.25 and consequently tend to be highly negatively charged above this value [9]. 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.

Effect of ionic strength on emulsion stability

The previous observations indicate that electrostatic repulsions play 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 (Figure 8). 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

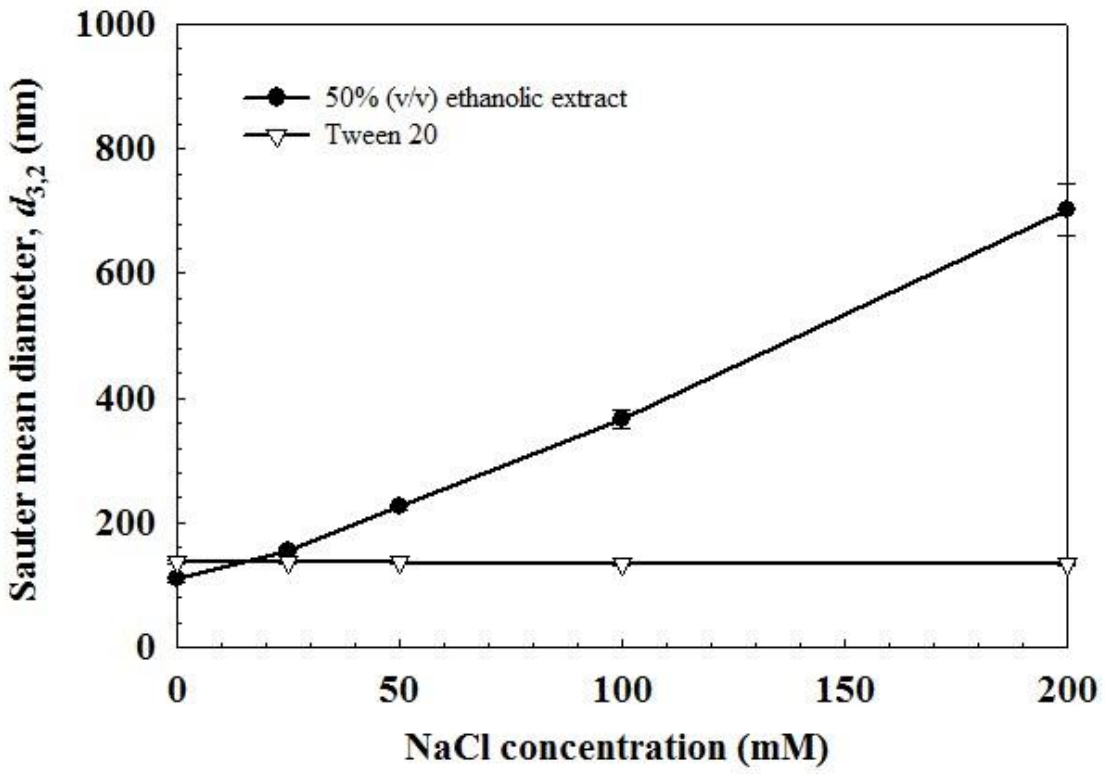


Figure 8. Effect of ionic strength on the $d_{3,2}$ of 10% (w/w) soybean O/W emulsions containing 2% (w/w) of Tween 20 or 50% (v/v) ethanolic extract.

non-ionic emulsifier, while emulsions containing 50% (v/v) ethanolic extract are exclusively stabilized because of the electrostatic repulsions between oil droplets.

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 7) 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 [51]. Fifty percent (v/v) ethanolic extract on the other hand contains surface-active species that strongly ionize at neutral pH thus providing sufficient electrostatic repulsions between droplets.

In-vitro gastric digestion of O/W emulsions

In this section, we evaluated the physical stability of prepared emulsions within the gastrointestinal tract conditions. The initial emulsions had small particle sizes (Figure 9) and uniform microstructures (Figure 10) as previously obtained. However, upon passage through the gastric phase, major changes were observed. Argan-based emulsions were appreciably increased in droplet size (Figure 9) and showed evidence of coalescence by the optical microscope images (Figure 10). Tween 20 emulsions, on the other hand, were very stable at stomach conditions, highlighting the non-ionic nature of this emulsifier. These results can be attributed to many factors

Table 6: 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			Tween 20		
	Day 1	Day 30	Day 60	Day 1	Day 30	Day 60
5 °C	110 nm	104 nm	118 nm	114 nm	120 nm	119 nm
25 °C	114 nm	113 nm	118 nm	113 nm	121 nm	115 nm
45 °C	113 nm	132 nm	146 nm	114 nm	121 nm	114 nm

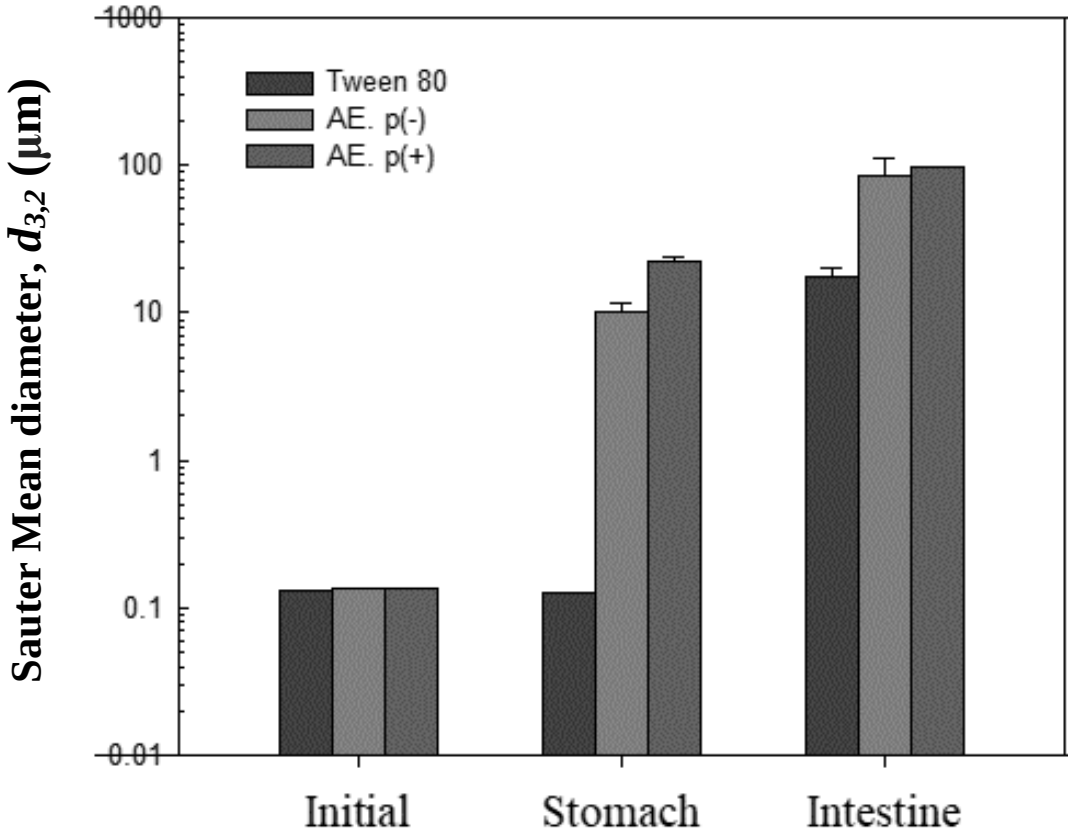


Figure 9. Effect of simulated gastrointestinal conditions on the Sauter mean diameter ($d_{3,2}$) of emulsions prepared using argan extract. AE. p(+) refers to argan extract-based emulsions digested in the presence of pepsin. AE. p(-) refers to argan extract-based emulsions digested in the absence of pepsin.

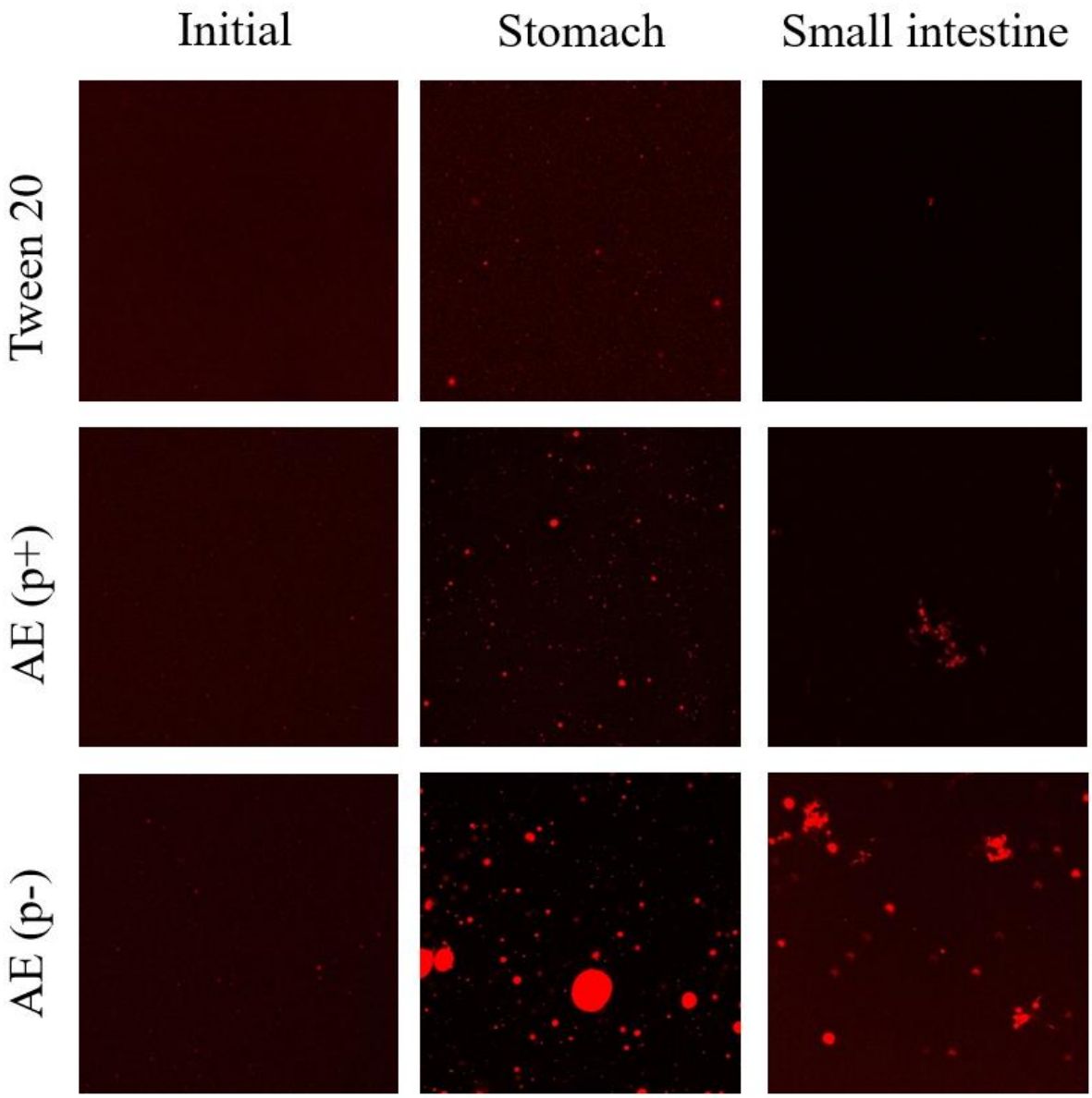


Figure 10. Effect of simulated gastrointestinal conditions on the microstructure of emulsions prepared using argan extract. AE. p(+) refers to of argan extract-based emulsions digested in the presence of pepsin. AE. p(-) refers to of argan extract-based emulsions digested in the absence of pepsin.

previously reported in literature, particularly: (i) the reduction of the electrostatic repulsion between oil droplets, (ii) the hydrolysis of the adsorbed protein on the droplet interface by pepsin and/or (iii) the promotion of depletion/flocculation phenomena under acidic conditions [52]. Interestingly, the addition of pepsin during digestion resulted in increased instability of argan-based emulsions as compared to the tother samples (Figures 9 and 10). We suggest therefore that proteins play a major role in the emulsifying ability of this extract and their digestion by pepsin contributed primarily to the observed instability within the gastric conditions.

Lipid digestion occurs within the small intestine, resulting in the release of FFA. We examined therefore the influence of digestion conditions on the formation of FFA and the physical characteristics of the prepared emulsions. As shown in Figure 9, all samples contained large particles, with broad particle size distributions and visible sedimentation (data not shown). These observations are probably due to the formation of calcium salts sediments or non-hydrolyzed oil aggregates, during digestion [53]. The microscopy images also indicated the presence of these larger particle, particularly when pepsin was added to the digestion system (Figure 10). This explains the reduced FFA release as compared to other samples (Figure 11), indicating the important role of proteins in the stability of argan-based emulsions.

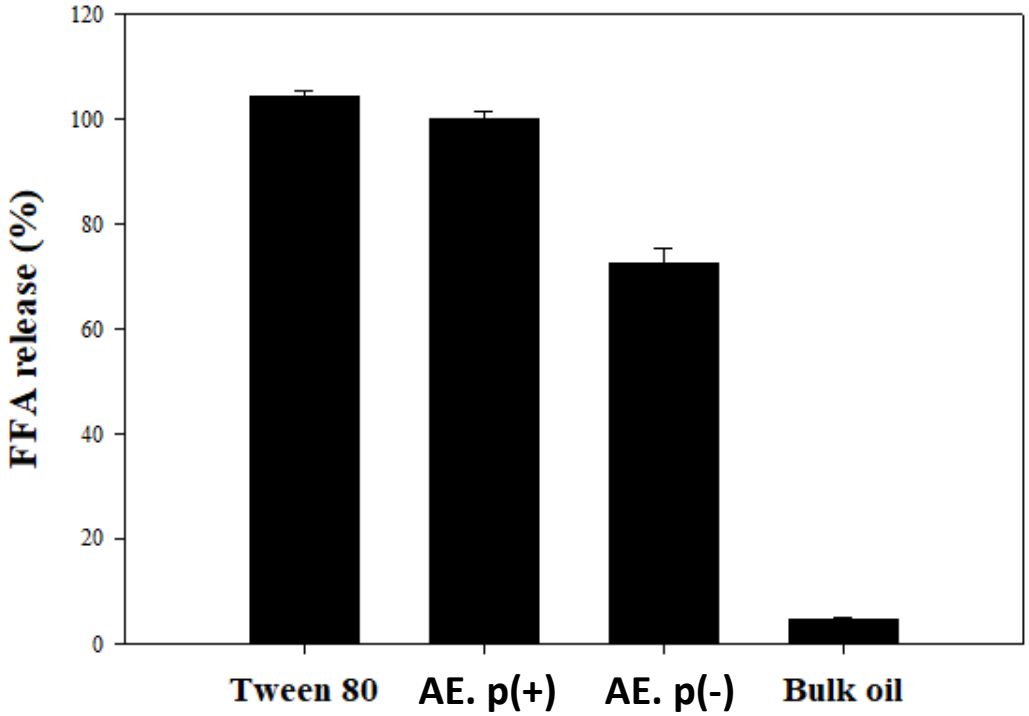


Figure 11. Effect of simulated gastrointestinal digestion on FFA release of emulsions prepared using argan extract. AE p(+) refers to argan extract-based emulsions digested in the presence of pepsin. AE. p(-) refers to argan extract-based emulsions digested in the absence of pepsin.

Chapter 3

Formulation characteristics of monodisperse O/W emulsions using a new natural emulsifier: Argan oil press-cake extract

Abstract

The complex composition of argan extract imparted its emulsifying efficiency, by creating a hydrophobic, or slightly hydrophilic, layer on the MC array plate surface. This resulted in unsuccessful emulsification using short MCs but did not affect the emulsification efficiency in longer ones. Using these longer channels, we could produce monodisperse O/W emulsions, with similar droplet size (~ 36 μm) and droplet size distribution (RSF < 0.25) to those obtained by Tween 80, and for up to 10 h of continuous emulsification. Our results indicate that non-purified emulsifying mixtures such as argan extract could be suitable for the preparation of monodisperse emulsions using MCE, by simple control of the emulsification system. In future, this could be useful for the production of entirely green label products using this technique.

This chapter will be submitted as:

Taarji, N, Vodo, S., Hafidi, A., Kobayashi, I., Marcos, A.N., Isoda, H. & Nakajima, M. (In preparation). The emulsifying performance of a surface-active extract from argan by-products in microchannel emulsification.

Introduction

A new trend in the utilization of emulsifiers suggests the use of less intensive practices for a sustainable production of emulsions. Particularly, the use of crude plant emulsifying extracts, obtained via simple extraction/separation steps, have been largely considered in recent times. Ralla et *al*. [50] evaluated the emulsifying properties of a crude extract from red beet by-products. They found that concentrations as low as 0.75% (w/w) of this extract can produce stable O/W emulsions using high-pressure homogenization. Other findings also include the good emulsifying properties of mildly derived extracts from oat bran, mullein and sunflower seeds [49, 53, 55]. This is an intriguing point regarding the necessity of using pure emulsifiers, as these studies show that multicomponent extracts can be successfully used for efficient stabilization of emulsions. We also found that a crude surface-active extract from argan press-cake can produce stable O/W nanoemulsions with similar stability and physical properties to those prepared using conventional emulsifiers such as Tween 20 [46]. In all these cases however, emulsions were prepared using high-energy emulsification methods, in order to increase the chances of obtaining stable emulsions. On the other hand, the effect of multicomponent extracts on the performance of low-energy emulsification methods, such as microchannel emulsification (MCE), is still unknown.

MCE is a microfluidic technique that enables the obtention of monodisperse emulsions using microfabricated channels, embedded on a silicon chip [56]. It is a particularly promising for the encapsulation of bioactive compounds, with superior physicochemical stability, as compared to conventional emulsification methods [57]. The technique is based on a simple process that demands no mixing or shear processing. Briefly, the dispersed phase is pressurized through the channels and the droplets are spontaneously formed (in less than 0.01 s) by the effect of interfacial tension. During this time however, the emulsifier should rapidly adsorb onto the droplets' interface,

i) to cause the distortion of the dispersed phase, allowing the formation of new droplets, and ii) to prevent newly generated droplets from coalescence [58]. Therefore, factors such as the speed of adsorption of surface-active components at the oil/water interface, their extent at reducing the interfacial tension, as well as their ability to spontaneously form a continuous film around the droplets, are crucial in MCE [59].

In this study, we evaluated the emulsifying performance of a surface-active extract from argan by-products in MCE. Our hypothesis is that the formation of monodisperse O/W emulsions is thermodynamically favorable using this extract, due to its high interfacial-activity [46]. However, its complex nature may also affect the emulsion formation behavior, which may require suitable conditions for the obtention of stable emulsions using this technique.

Materials and methods

Materials

Argan press-cake was kindly provided by an oil-producing cooperative in the region of Essaouira, Morocco. According to the manufacturer, the cake was obtained by a simple process that involves no heat treatment or further extraction of residual oil. Ethanol (99.5%), acetonitrile (HPLC grade), formic acid (HPLC grade), refined soybean oil and polyoxyethylene (20) sorbitan monooleate (Tween 80) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Whatman paper was obtained from GE Healthcare Life Sciences (Chicago, Illinois, USA). Disposable syringe filters (0.22 μm pore size, Nylon) were obtained from Advantech Co., Ltd. (Taipei, Taiwan). Milli-Q water with a resistivity of 18 MΩ·cm was produced by an ultrapure water system (Arium® pro, Goettingen, Germany*)* and used to prepare all solutions in the current study.

Preparation of Argan extract

Argan press-cake was initially grinded into small particles (< 0.5 mm) using a high-speed mixer. The obtained powder (100 g) was then added to a fresh mixture (500 mL) of water and ethanol (20/80, v/v) and extracted for 2 hours at room temperature. After centrifugation (3000 rpm for 30 min) and filtration (Whatman paper) to remove solid particles, the solvent was then evaporated under reduced pressure to give a syrupy residue that was re-dispersed in water and re-filtered using a 0.22 μm syringe filter. Finally, for an accurate preparation of working solutions and to prevent microbial growth during storage, the extract was freeze-dried for 3 days at -80 °C and 5 Pa (Eyela, FDU 2110, Rika Kikai *Co*., Ltd., Tokyo, Japan), and kept at 5 °C until use.

Physicochemical characterization of argan extract

Total proteins and saponins contents were previously described in Taarji et *al*. [46]. Herein, the presence of glucuronic-type saponins was analyzed by means of mass spectrometry (LCMS-8050, Shimadzu Ltd., Kyoto, Japan), following the method of Henry et *al*. [25]. Briefly, a 10 μL aliquot of argan extract was injected into a C18 separation column (250 x 2.1 mm^2, 5 um, Shim pack, Shimadzu Ltd., Kyoto, Japan) and the compounds of interest were eluted using a linear 25 min gradient, from 30 to 60% acetonitrile in 0.1% formic acid, at 0.4 mL/min and 50 °C. The mass spectrometer operated then in the negative ion electrospray ionization mode ESI(-), acquiring a full scan from 1000-1500 m/z.

Viscosity was determined using a vibro-viscometer (SV-10, A&D Co., Ltd., Tokyo, Japan) at 25 ℃, by measuring the electric current needed to resonate through the solution. Particle size and surface charge were measured using a ς-analyzer (Zetasizer, Nano ZS, Malvern Instruments Ltd., Worcestershire, UK), over 10–100 runs per cycle. Finally, contact angle was measured according

to the method of Zhang et *al*. [60], by measuring the angle of generated drops to the plasma oxidized microchannel (MC) plate surface (PD-W, Kyowa Interface Science Co., Ltd., Saitama, Japan).

Microchannel array chips

Asymmetric straight-through silicon MC array chips with different channel dimensions were used in this study. The chips were fabricated through a repeated process of photolithography and deep reactive etching by Hitachi Power Semiconductor Device, Ltd., and commercialized by EP Tech, Co., Ltd. under different models (e.g. WMS 1-2).

Figures 12a and 12b depict the structure of WMS 1-2 and WMS 1-3 chips, respectively. Both chips measure 24 x 24 x 0.5 mm^3 and include a 100 μm-etched active area, containing MCs. Each MC consists of a 10 x 70 μm^2 cylindrical microhole on the bottom side and a rectangular microslot on the top side. However, WMS 1-3 contains 23 348 MCs, while WMS 1-2 contains only 10 313 MCs. This is because the microslots in WMS 1-2 (10 x 100 x 30 μm^3) are wider than those in WMS 1-3 (10 x 50 x 30 μm^3), which results in less channels on the horizontal line. In WMS 11-1 (Figure 12c), the active area is etched to only 200 μm, resulting in longer microholes (13 752 MCs). Here, each MC consists of a 10 x 160 μm^2 microhole and a 10 x 80 x 40 μm^3 microslot.

Before conducting experiments, the chips were ultasonicated at 100 KHz with neutral detergent, disinfectant ethanol and water, respectively. They were then subjected to plasma oxidation (PR500, Yamato Science Co. Ltd., Tokyo, Japan), to develop a hydrophilic layer on the surface.

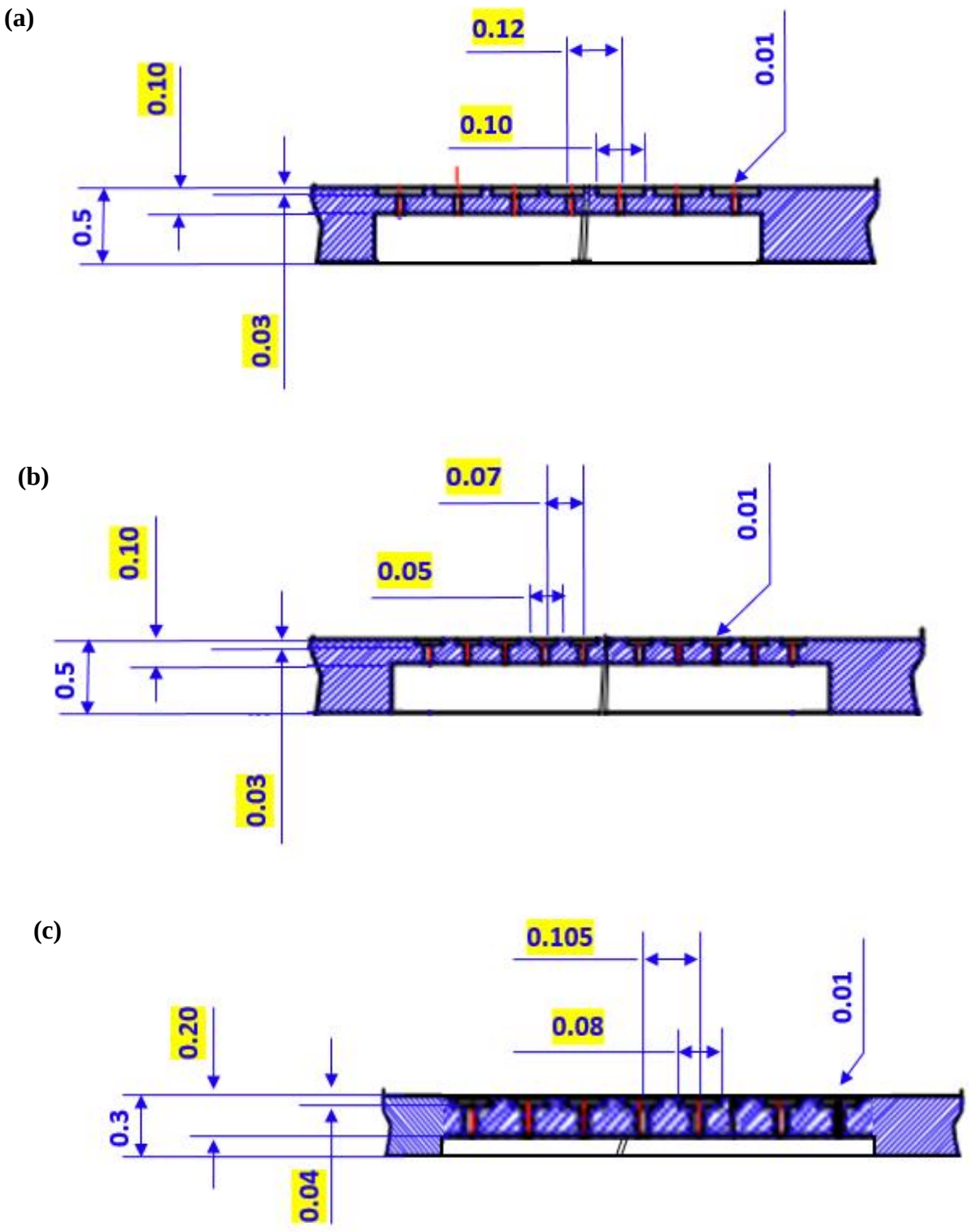


Figure 12. Cross-sectional representation of MC array chips used in this study: (a) WMS 1-2, (b) WMS 1-3 and (c) WMS 11-1. Variable dimensions (mm) are highlighted in yellow.

Emulsification procedure

Emulsions were prepared at room temperature as illustrated in Figure 13. First, the MC chip was mounted in the emulsification module, with the microslots facing the top. Next, the oil was injected to the bottom side of the chip, using a programmable syringe pump (Model 11 Elite, Harvard Apparatus Inc., Holliston, USA), at 2 mL h^{-1}. When the droplets started to generate, the continuous phase (1% w/w) was finally delivered, from an elevated reservoir, to the top side of the chip, which enables the recovery of generated droplets.

Droplet characterization

During emulsification, the droplet generation behavior was recorded using a CCD microscope video system (MS-511B, Seiwa Kougaku Sesakusho Ltd., Tokyo, Japan). After emulsification, the droplet size was measured using a laser diffraction particle size analyzer (LS 13,320, Beckman Coulter, Brea, USA) and the droplets were observed using an optical microscope (Leica DFC 7000 T, Wetzlar, Germany). The average droplet size was expressed as sauter mean diameter $d_{3,2}$, while the droplet size distribution was presented by relative span factor (RSF):

$$RSR = d90 - d10 \,/\, d50$$

Where d_{90}, d_{50} and d_{10} are the respective intercepts for 90, 50 and 10% of the cumulative mass.

Results and discussion

Physicochemical characteristics of argan extract

Argan extract was prepared using a simple solvent extraction procedure, without fractionation or purification of the obtained composition. It is therefore a mixture of multiple compounds that could mutually affect the surface-active and/or emulsifying properties, as compared to individual ingred-

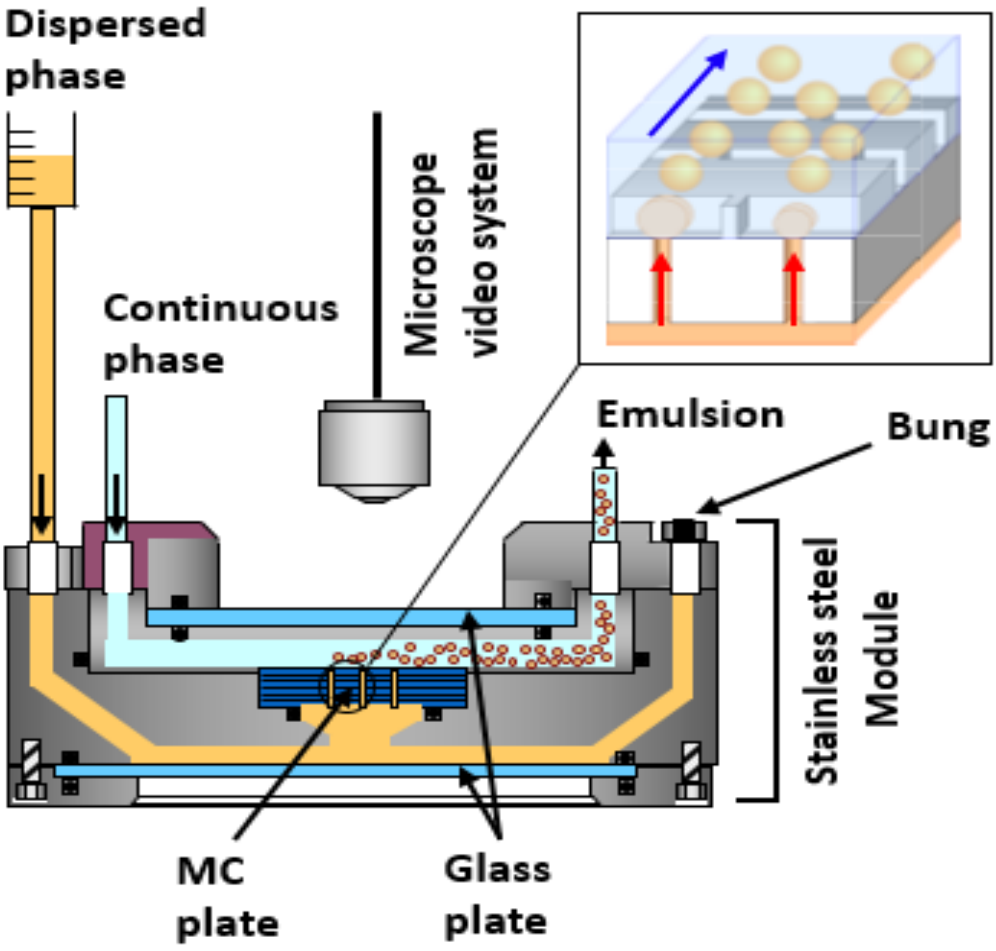


Figure 13. Schematic representation of MCE setup used in this study.

ients. Earlier investigation about the composition of this extract indicated the presence of around 3.5% (w/w) of triterpenoid saponins (Figure 2). These compounds have an amphiphilic chemical structure and a relatively low molecular weight, thus acting as small molecule surfactants [9]. The use of triterpenoid saponins have been shown previously to reduce the interfacial tension and to produce stable O/W emulsions using high-energy emulsification. Nevertheless, a relatively high concentration of saponins is usually required to produce smaller droplet size emulsions, as compared to commonly used surfactants [14]. Recent applications of saponins in O/W emulsions, suggest therefore their use in mixed surfactant systems, for improved surface-active and/or emulsifying properties. Reichert et *al*. [40] for example found that the concentration of Quillaja saponins could be successfully reduced, without changes in emulsion droplet size or droplet size distribution, following the addition of limited amounts of sodium caseinate. Other findings also include the synergetic effect of saponins and proteins in reducing the interfacial and surface tensions [61]. In general, it is suggested that the presence of saponins and proteins in mixed surfactants systems results in molecular complexations that facilitate their adsorption at the oil/water or air/water interfaces [62, 63].

In our previous study, we could also detect the presence of a lower concentration of proteins in the present extract (1.1% (w/w)) [46]. However, it was not clear how the mutual presence of saponins and proteins could result in the formation of biogenic complexes that could explain the observed interfacial activity (Table 8) at these very low concentrations. In literature, the molecular complexation of saponins and proteins is usually explained by the action of low molecular forces such as hydrogen bonds, hydrophobic and electrostatic interactions. Wojciechowski et *al*. [64] for example suggested that the presence of deprotonated glucuronic acid in the molecules of Quillaja saponins could result in electrostatic attractions with the positively

Table 7. Physicochemical characteristics of argan extract and Tween 80 solutions (1%, w/w).

	Argan extract	**Tween 80**
Interfacial Tension* (mN m^{-1})	11.5 ± 0.3	5.8 ± 0.3
ς-potential (mV)	-51.1 ± 2.0	-4.3 ± 1.6
Particle size (nm)	133.9 ± 0.5	9.4 ± 0.1
Contact angle (°)	52.4 ± 4.2	19.1 ± 2.1
Viscosity (mPa.s)	0.95 ± 0.2	0.91 ± 0.0

charged domains in lysozyme proteins. Similar behavior was also suggested in Quillaja saponins/β-casein and Quillaja saponins/β-lactoglobulin systems [61, 63]. In the present study, we could also identify several glucuronic-type saponins in our extract (Figure 14), by comparison to the previously obtained ESI(-)-MS chromatograms by Henry et *al*. [25]. These compounds have an acidic pKa of around 3.25 and therefore tend to be completely deprotonated at neutral pH [9], which can result in electrostatic bindings with proteins.

The formation of proteins/saponins complexes may occur either in bulk solution, prior to adsorption, or directly at the interface, through co-adsorption of the slower diffusing component on the pre-adsorbed layer of the other component [64]. To gain more insight about the structural organization of surface-active components in our extract, we measured the particle size and the particle size distribution of the prepared solutions. As shown in Table 8, argan extract has an average particle size of around 133 nm, while Tween 80 resulted in a smaller size of 9.4 nm. Normally, small molecule surfactants such as Tween 80 and saponins are expected to result in smaller micellar structures, in comparison to proteins or proteins/saponins complexes. Mitra & Dungan [15] for example found that the size of Quillaja saponins micelles generally ranges from 6-12 nm. Tween 80 micelles size should be also in that range. In our extract however, the particle size measurement resulted in a single pic that only starts from around 50 nm (Figure 15). This means that no smaller micellar formations were present in this solution and therefore saponins were exclusively adsorbed on larger aggregates and/or present in free soluble form.

Evidences presented in this study indicate that argan extract contains relatively large aggregates, which are likely to result from electrostatic interactions between glucuronic-type saponins and proteins. Interestingly, the ς-potential measurement of this extract indicated a strong negative charge of about -51 mV (Table 8). It is difficult to determine the exact source of this charge.

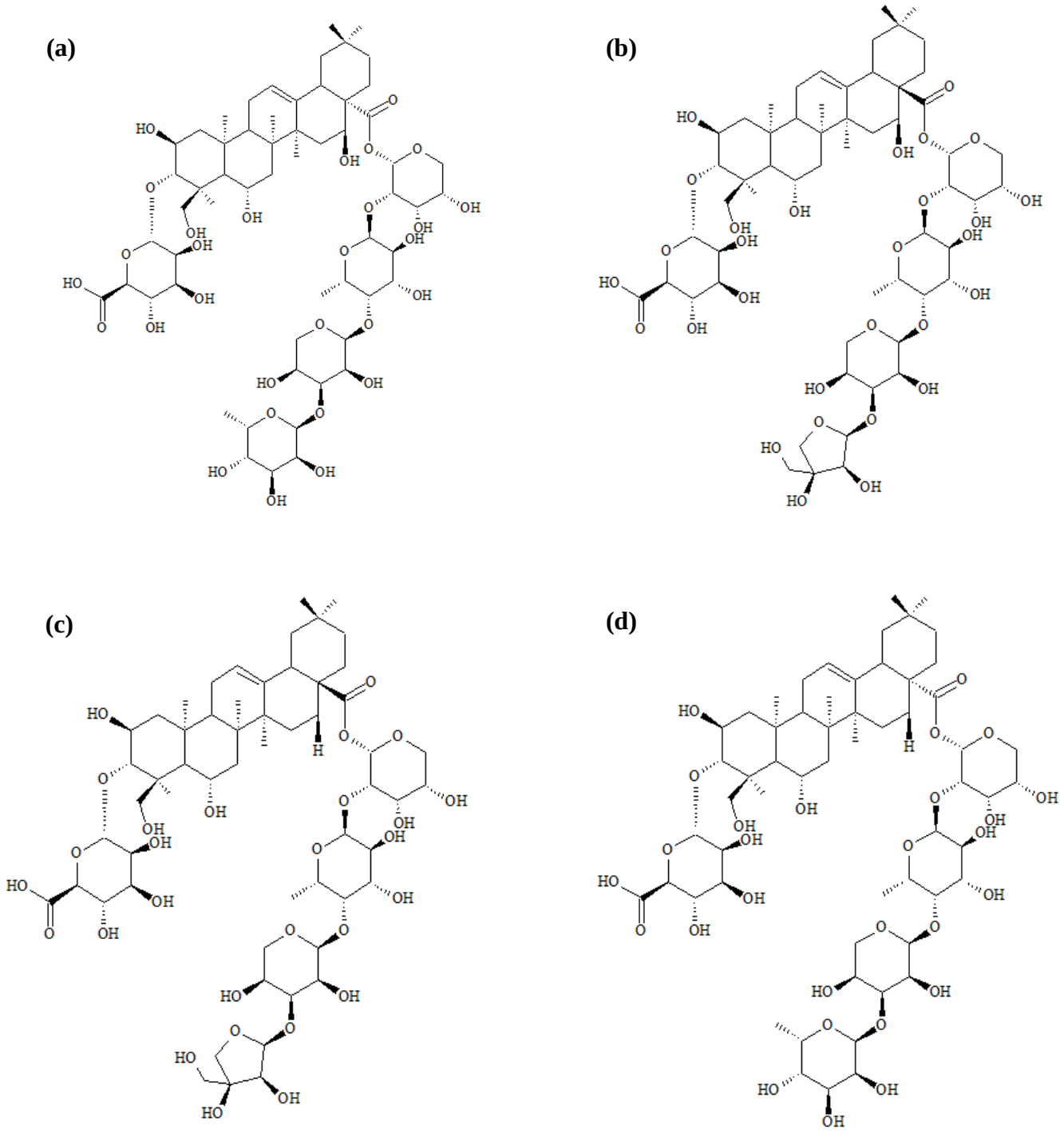


Figure 14. Chemical representation of glucuronic-type saponins identified in argan extract. (a) m/z = 1251.6 (b) m/z = 1237.6 (c) m/z = 1235.6 and (d) m/z = 1221.6.

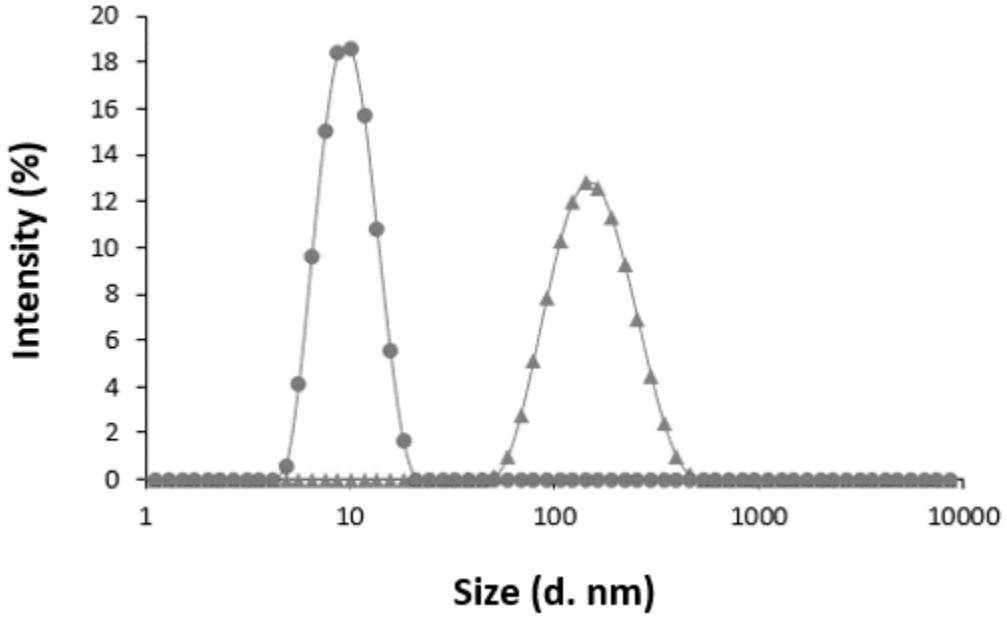


Figure 15. Particle size distribution of argan extract and Tween 80 solutions (1% w/w). (•) refers to Tween 80, (▲) refers to argan extract.

However, since ς-potential is the measurement of the surface charge of colloidal particles in dispersion, we correlate this charge to the components of the previously identified aggregates. Normally, upon depletion of the positively charged domains in the protein structures, we can also imagine the formation of other non-covalent bindings with saponins. Böttcher et *al*. [62] for example hypothesized that in binary systems, saponins and proteins can interact together by hydrogen bonds and/or hydrophobic interactions, forming biogenic complexes. Therefore, in our speculation, this strong negative charge in argan extract could be due to the glucuronic-type saponins that are non-electrostatically adsorbed to the complex aggregates and/or to the negatively charged domains in the protein structures themselves. Actually, in our previous study, we also found that emulsions prepared using a similar extract from argan press-cake, by high-pressure homogenization, were very sensitive to acidic pH, resulting in the loss of droplets negative charge and physical stability of emulsions [46]. However, even at pH 3, providing that the pKa of glucuronic-type saponins is around that pH [9], the droplets still had a relatively high negative charge on their surface, which indicates that saponins might not be the only contributors to this charge [46].

Overall, the composition and interfacial activity of argan extract seems to be favorable for successful MCE. The formation of proteins/saponins complexes is very favorable and this is believed to provide improved emulsifying properties to this extract, as compared to individual ingredients. Other factors, such as the low viscosity (Table 8) and the relatively small particle size (as compared to MCs microhole diameter) are also believed to acquire a smooth droplet generation during MCE, by allowing a fast inflow of the continuous phase, as compared to the outflow of the oil phase [65].

Droplet generation behavior in MCE

MCE was introduced to the first time by Kawakatsu et *al*. [66], as a microfluidic technique for the preparation of monodisperse emulsions using microscopic channels, embedded on a silicon chip. Since that time, various MC designs and geometric structures have been fabricated and evaluated for their efficiency using this technique. Nowadays, asymmetric straight-through MC arrays are the most commonly used devices in MCE. They are particularly robust at carrying stable droplet generation using high dispersed phase flow rates and low viscosity oils, hence being more promising for mass and economical production of monodisperse emulsions [57]. In this section, we describe the emulsifying performance of argan extract in three different asymmetric straight-through MC array chips, with various microhole lengths and microslots widths. Clear observation of the emulsification behavior was possible thanks to the clarity of argan extract (Figure 16).

As shown in Figures 17a and 17b, droplet generation in WMS 1-2 and WMS 1-3 was not successful using argan extract as emulsifier. The dispersed phase that passed through the working channels started to inflate rapidly along the plate surface, until neighboring droplets contacted each other and aggregated into larger particles. At this point, it was not clear whether this behavior results from a limited concentration of the emulsifying compounds in the system, to the interaction between the plate surface and the extract composition or simply to a defect in the MCs design. We conducted therefore new experiments using different MCs, with longer microholes, and compared the droplet generation behavior to the commonly used surfactant Tween 80. As shown in Figure 17, Tween 80 solution exhibited a smooth droplet generation behavior, regardless of the MCs dimensions. On the other hand, argan extract was only successful in WMS 11-1, provided that the same concentration (1% w/w) was used in all experiments. Sugiura et *al*. [58] previously showed that narrow and longer channels result in better droplets generation in MCE. It was therefore

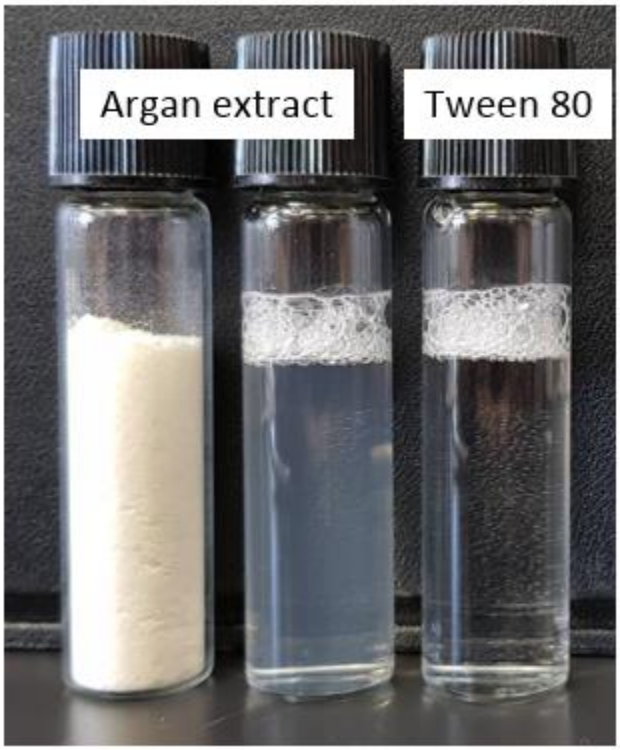


Figure 16. Visual appearance of dried and dissolved argan extract (1%, w/w).

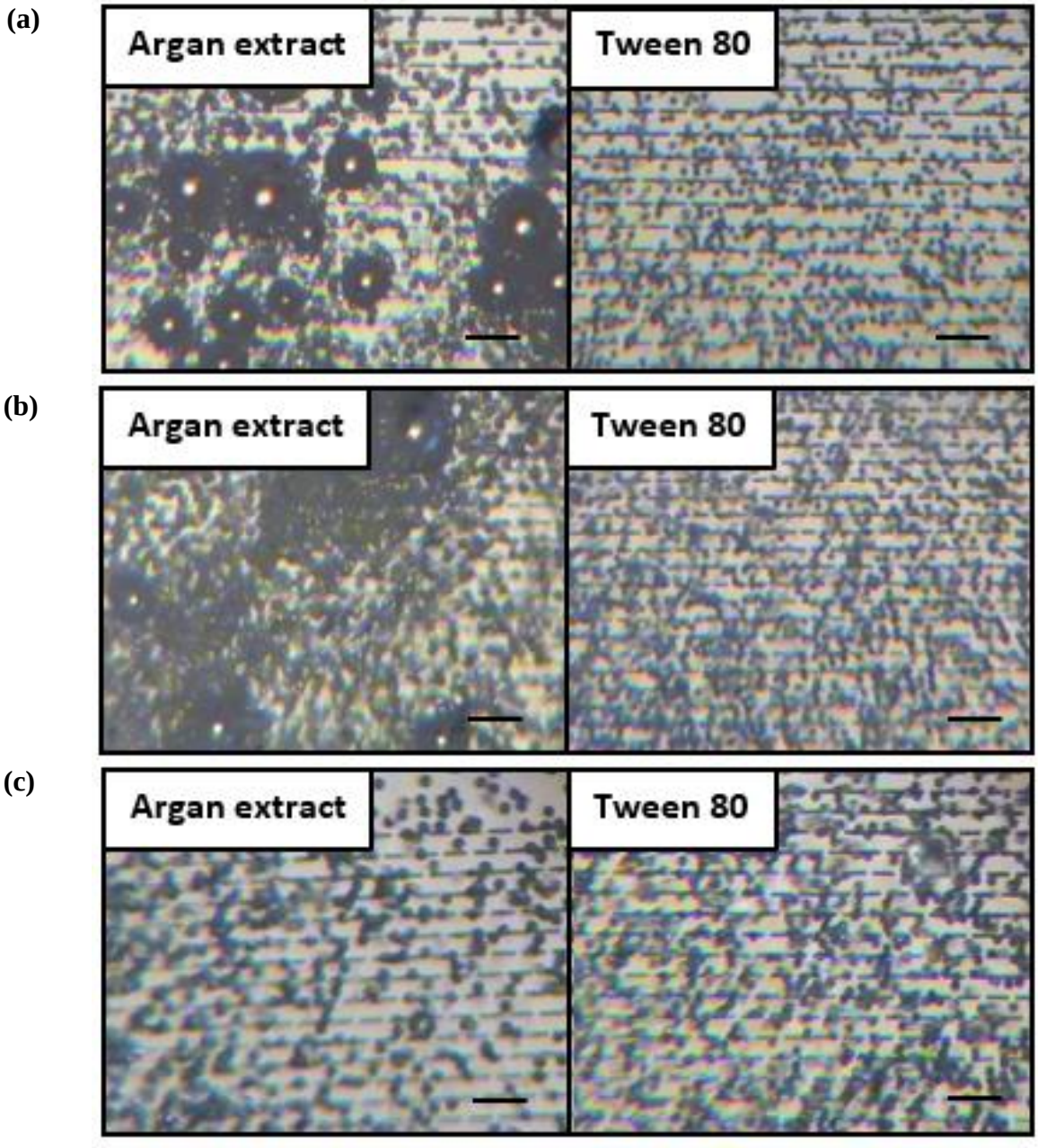


Figure 17. Effect of emulsifier type (1% w/w) on droplet generation behavior in (a) WMS 1-3, (b) WMS 1-2 and (c) WMS 11-1; bar = 200 um.

expected that longer channels in WMS 11-1 would provide better emulsification properties than shorter channels in WMS 1-2 and WMS 1-3. However, these latter were still proven to function correctly when using Tween 80, which suggest that other factors, indigenous to the extract composition, were responsible for the droplets inflation behavior that was observed in these short MCs.

Saito et *al*. [59] evaluated the emulsifying performance of various proteins in MCE. They found that when the pH was lower than the isoelectric point of the protein, electrostatic attractive forces worked between the negatively charged silicon MC plate and the positively charged protein molecules, resulting in unsuccessful emulsification. In our case, we previously showed that argan extract contains negatively charged particles (Table 8) that should normally repulse with the silicon surface. However, upon measurement of the contact angle, we found that argan extract interacts actually more with the MC chip surface, in comparison to Tween 80 (Table 8). This means that other components of this extract have higher affinity to the chip material, thus resulting in higher contact angles. In general, a higher contact angle means that the emulsifier solution yields a hydrophobic, or less hydrophilic, layer on the chip surface. A lower contact angle on the other hand means that the previously oxidized chip was less affected by the emulsifier solution composition, thus maintaining its original hydrophilic surface [60]. In the present extract, it was difficult to determine the exact compounds that could interact with the MC array chip surface. However, since the contact angle was increased (52.4°), as compared to Tween 80 (*19.1°)*, we speculate that these compounds have an amphiphilic structure, with the polar region binding to the silicon dioxides on the chip surface and the hydrophobic regions facing the chip exterior, thus creating a hydrophobic layer on the surface.

Tong et *al*. [67] and Kobayashi et *al*. [68] initially emphasized the importance of hydrophilic MC array plates for successful production of O/W emulsions in MCE. Consequently, plasma-oxidized chips are normally used for the production of O/W emulsions, to avoid wetting of oil phase on the chip surface. Nevertheless, it has been shown previously that the slightest reduction in the hydrophilicity of the chip could greatly affect the particle formation behavior in MCE [59-60]. Using argan extract, this resulted in unsuccessful emulsification in WMS 1-2 and WMS 1-3 chips. This malfunctioning however could be easily repaired by using longer channels in WMS 11-1, providing monodisperse O/W emulsions with similar droplet size (~ 36 μm) and droplet size distribution (RSF < 0.25) to those obtained by the commonly used surfactant Tween 80 (Figure 18).

Long-term production stability of O/W emulsions

The previous observations indicate that argan extract interacts more with the MC array chip by creating a hydrophobic, or slightly hydrophilic, layer on the surface. This resulted in unsuccessful emulsification using WMS 1-2 and WMS 1-3 chips, but interestingly did not impact the droplet generation behavior in longer MCs of WMS 11-1. In this section, we evaluated the long-term production stability of O/W emulsions prepared using argan extract, to assess the possible deposition of such a layer over time, thus affecting the emulsification efficiency.

As shown in Figure 19, the droplet size and droplet size distribution were almost unchanged over the entire period of emulsification. The droplets were uniformly sized and spherical and were easily swept-away by the continuous phase cross-flow, without interaction with the chip surface. Tong et *al*. [67] initially demonstrated that stable droplet generation could be successfully maintained for up to 4 h when using MCE. Vladisavljević et *al.* [69] also reported no change in

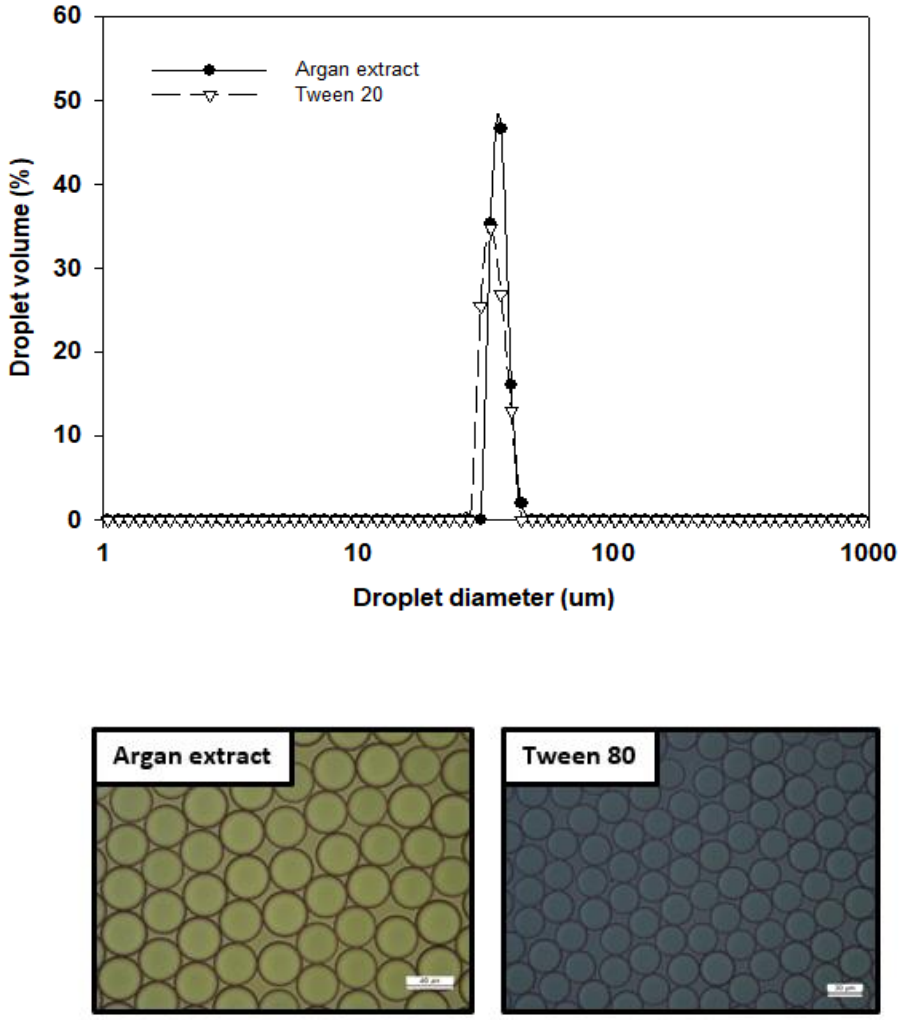


Figure 18. Droplet size distribution and optical micrographs of prepared emulsions in WMS 11-1 using 1% (w/w) argan extract or Tween 80.

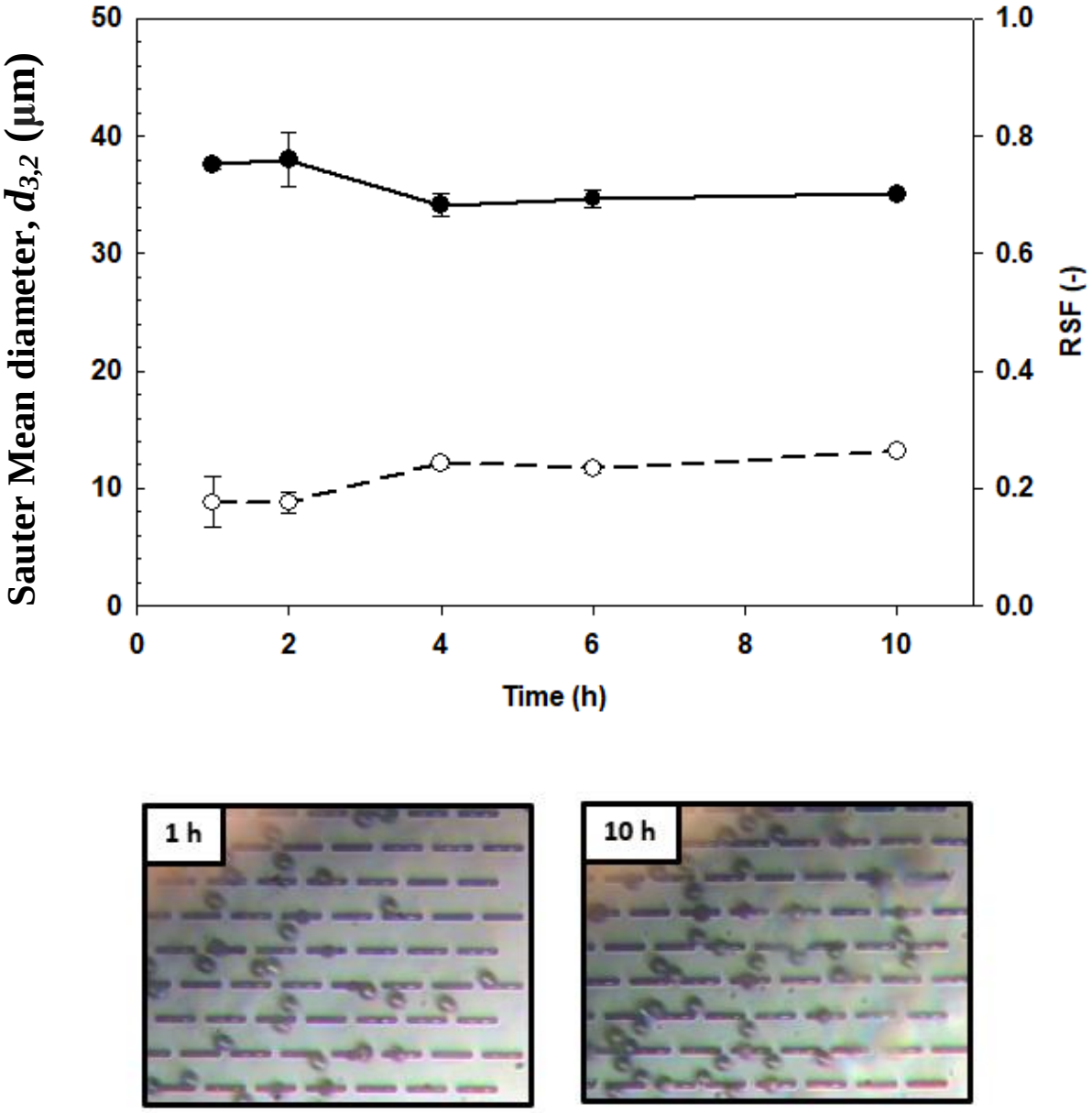


Figure 19. (a) Droplet size ($d_{3,2}$) and RSF of emulsion prepared using argan extract (1% w/w), during long-term MCE. (b) Droplet generation behavior after 1 and 10 h of continuous emulsification.

droplet generation characteristics after 7 h of continuous emulsification. In general, it seems that MCE technology is very suitable for long-term production of monodisperse emulsions. However, it has been shown previously that the choice of emulsifier could play a key factor in continuous operating of MCE. Tween 20 for example was found to gradually increase the affinity between the oil phase and the chip surface, thus resulting in variations of the droplet generation behavior and the droplet size distribution. SDS on the other hand presented a smooth droplet generation in continuous MCE, by providing strong repulsive forces between the generated droplets and the chip surface [70]. To assess the possible interaction between the MC chip surface and the generated droplets using argan extract, we measured the ς-potential of prepared emulsions. As expected, emulsions prepared using argan extract presented a highly negative droplet charge of around -65 mV (data not shown), in accordance to the previously identified particles in Table 8. We suggest therefore, that during emulsification using this extract, the generated droplet strongly repulsed with the MC array chip surface, due to the negatively charged coating provided by surface-active components of this extract. This allowed to maintain a smooth droplet generation behavior over the entire period of emulsification, without variation in droplet size or droplet size distribution. Meanwhile, long channels in WMS 11-1 were necessary for a successful droplet formation to occur in the first place, potentially by creating a large pressure drop between the MCs chamber and the chip surface, thus facilitating the droplet detachment on the outer edge of the MC [58]

Chapter 4

The emulsifying performance of Glycyrrhizin (saponin) and a non-purified glycyrrhizin-rich extract from licorice root: Insights to the emulsifying mechanism of multicomponent plant extracts

Abstract

In licorice root extract, glycyrrhizin molecules are likely attached to other surface-active components, such as proteins, which results in improved interfacial activity and emulsion forming ability as compared to purified glycyrrhizin. The emulsions were superiorly stable at low pH and high salt concentration but less stable at elevated temperatures indicating the effective contribution of proteins to the observed emulsifying performance. Evidences presented in this study indicate that non-purified surface-active extracts, such as argan and licorice root extracts, can provide superior emulsifying ability in comparison to purified emulsifiers. This is due to the contribution of multiple compounds to the surface-active properties and the formation of biogenic complexes between these compounds, thus leading to improved droplet coverage and surface-activity.

This chapter will be submitted as:

Taarji, N, Bouhoute, M., Chafai, Y., Hafidi, A., Kobayashi, I., Marcos, A.N., Isoda, H. & Nakajima, M. (In preparation). Preparation characteristics of O/W nanoemulsions using glycyrrhizin and a non-purified glycyrrhizin-rich extract from licorice (*Glycyrrhiza glabra*) root.

Introduction

Emulsions properties are largely influenced by the type of emulsifier(s) used during homogenization. First, they tend to reduce the interfacial tension between oil and water phases, thus enhancing droplet disruption [71]. They also form a protective layer around the newly generated droplets, which prevent them from coalescence [72]. Selecting the right emulsifier(s) is therefore necessary to provide established characteristics to the final product. Particularly, the size, stability and rheological properties of emulsions can be carefully controlled by changing the type of emulsifier(s) [7].

Many emulsifiers are currently available for the preparation of oil-in-water (O/W) emulsions. Natural surfactants, such as saponins and lecithin are usually applied to produce small droplet size emulsions (≤ 200 nm). They are composed of distinct lipophilic and hydrophilic regions, which provide them with high affinity to droplet interface [73]. Surface-active biopolymers, such proteins and polysaccharides are also utilized to produce O/W emulsions. They can provide better stability attributes in comparison to small molecular surfactants, but often require a high emulsifier-to-oil ratio to produce fine emulsions [36]. A recent trend in the utilization of emulsifiers suggest therefore the use of mixed emulsifiers systems for the production of stable nanosized emulsions. Particularly, the use of protein-saponin binary systems have been of great interest [39].

Protein and saponins are originally present in many plant materials and do not have to be initially combined to be applied as emulsifiers. In our previous study, we found that a non-purified extract from argan press-cake could produce stable O/W nanoemulsions, using high-pressure homogenization [46]. Other findings also include the good emulsifying properties of sugar beet, oat bran, mullein and soybean seed crude extracts in O/W emulsions [49, 50, 55]. The main stabilizing mechanism is believed to the formation of biogenic complexes between proteins and

saponins at the oil/water interface [50]. However, it is still not clear how the formation of these complexes could result in improved emulsifying properties as compared to individual ingredients [39].

In this study, we evaluated the emulsifying performance of a saponin-rich extract from licorice root in O/W emulsions. We also evaluated the formulation characteristics of emulsions prepared using purified saponins (Glycyrrhizin) from the same origin. Our aim is to produce stable O/W nanoemulsions using this extract as sole emulsifier. We also hope to provide progressive insights about the emulsifying mechanism of multicomponent plant extracts in general.

Materials and methods

Materials

Licorice root powder was obtained from Biorigins Co. (Hampshire, UK). Glycyrrhizin (> 93%) was provided by Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ethanol (99.5%), soybean oil, sodium hydroxide and hydrochloric acid were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Whatman filter paper was obtained from GE Healthcare Life Sciences (Chicago, Illinois, USA). Disposable syringe filters (0.45 μm, hydrophobic PTFE membrane) were obtained from RephiLe Bioscience, Ltd. (Shuangbai, China). Milli-Q water, with a resistivity of 18 MΩ cm was produced by an ultrapure water system (Arium® pro, Goettingen, Germany) and used to prepare all solutions in the current study.

Preparation of licorice root extract

The samples (100 g) were added to fresh mixtures (500 mL) of distilled water and ethanol (25:75) and stirred for 2 hours at room temperature. The suspensions were then centrifuged and filtered

(0.45 μm) to remove solid particles, and the solvents were eliminated by rotary evaporation (40 °C, 16 hPa) and lyophilization (-80 °C, 4 Pa).

Physicochemical characterization of licorice root extract

Glycyrrhizin content was determined according to the method of Tiang et *al*. [76], by HPLC-UV analysis (LC-2000 plus, Jasco Co., Ltd., Tokyo, Japan). Protein content was determined using a total nitrogen analyzer (UNICUBE, Elementer Ltd., Yokohama, Japan), with a nitrogen-to-protein conversion factor of 6.25. Polyphenols content was spectrophotometrically determined following the method of Rabello et *al*. [77], using gallic acid as an external standard ($R^2 = 0.9992$). Interfacial tension was measured at the soybean oil/water interface, using an automatic pendant drop tensiometer (PD-W, Kyowa Interface Science Co., Ltd., Saitama, Japan). Particle size and surface charge were measured using a Dynamic Light Scattering/ ς-*p*otential Analyzer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). Viscosity was measured at 25 °C using a Stabinger viscometer (SVM 3001, Anton-Paar Ltd., Graz, Austria).

Preparation of O/W emulsions

Coarse emulsions were initially prepared by homogenizing appropriate amount of extract solution (1 wt.% in water) and soybean oil at 10000 rpm for 5 min (Polytron PT-3000, Kinematica Inc., Luzern, Switzerland). The obtained emulsions were then passed through a high-pressure homogenizer (NanoVater NV200, Yoshida Kikai Co., Ltd., Nagoya, Japan) at 100 MPa for four passes and stored at 25 °C until analysis.

Effect of pH on emulsions stability

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

Effect of salt on emulsions stability

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

Effect of Temperature on emulsions stability

Samples were incubated for 30 min at appropriate temperature (40-120 °C) in an preheated oil bath and let to cool down to room temperature before analysis.

Particle characterization

The microstructures of prepared emulsions were observed using an optical microscope at x40 magnification (DM IRM, Leica Microsystems Pty., Ltd., Wetzlar, Germany). The mean droplet size and droplet size distribution were measured using a static laser diffraction particle size analyzer (LS 13,320, Beckman Coulter, Brea, USA). The droplet charge was determined using a ς-potential analyzer (Zetasizer, Nano ZS, Malvern Instruments Ltd., Worcestershire, UK), following a 1:100 dilution with MQ-water.

Results and discussion

Physicochemical properties

Plant extracts are a mixture of multiple compounds that can exhibit surface-active and/or emulsifying properties. Saponins are usually investigated due to their amphiphilic and small-molecular characteristics. Other compounds such as proteins and polyphenols are also important and can contribute to the overall emulsifying ability [78]. Licorice root extract contained around 8% (w/w) of glycyrrhizin, 6.87% (w/w) of proteins and 6.72% (w/w) of polyphenols. The extract has a monomodal size distribution with a mean particle size of around 30 nm and a negative ζ-potential of -27.8 mV at pH 7. It also has a low viscosity, when dissolved in water (1%, w/w), and shows a strong interfacial activity at the oil/water interface (Table 2).

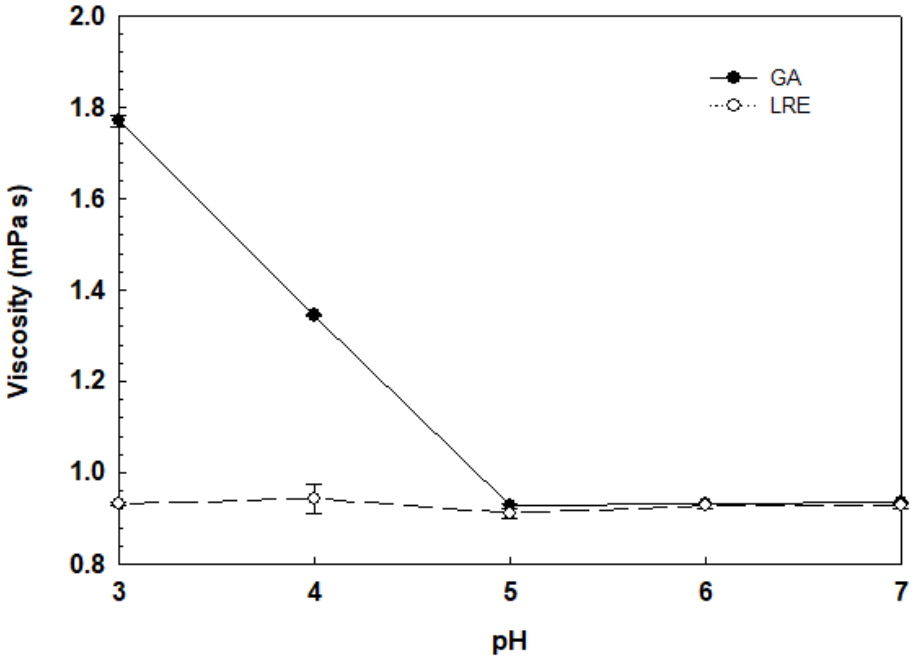
Interfacial tension of plays an important role in the formation and stabilization of O/W emulsions [22]. We investigated therefore the interfacial properties of licorice root extract and purified glycyrrhizin, at constant saponins concentration, to evaluate the contribution of specific compounds to the overall emulsifying ability. As shown in Figure 20a, licorice root extract was capable of reducing the interfacial tension at all pH values. Purified glycyrrhizin on the other hand was only surface-active at low pH. It reduced the interfacial tension from 26.2 mN m^{-1} to around 10.8 mN m^{-1}, but progressively lost this ability as we increased the pH.

Previous investigation about the properties of glycyrrhizin indicated the formation of self-aggregates at low pH. As we increase the pH, the molecules tend to dissociate due to the increased repulsion between adjacent molecules, resulting in reduced interfacial activity [79]. Interestingly, this phenomenon was not observed in licorice root extract, indicating that saponins are probably adsorbed to other compounds in bulk solution, thus reducing the repulsions at the oil/water interface.

Table 8. Surface-active composition and fluid characteristics (1 wt.% in water, pH 7) of licorice root extract.

Physicochemical property	
Glycyrrhizin (wt.%)	7.99 ± 1.28
Proteins (wt.%)	6.87 ± 0.44
Polyphenols (wt.%)	6.72 ± 0.47
Interfacial Tension (mN m^{-1})	12.0 ± 0.1
z-average particle size (nm)	29.12 ± 0.40
ζ-potential (mV)	-27.80 ± 1.45
Viscosity (mPa.s)	1.06 ± 0.04

a)



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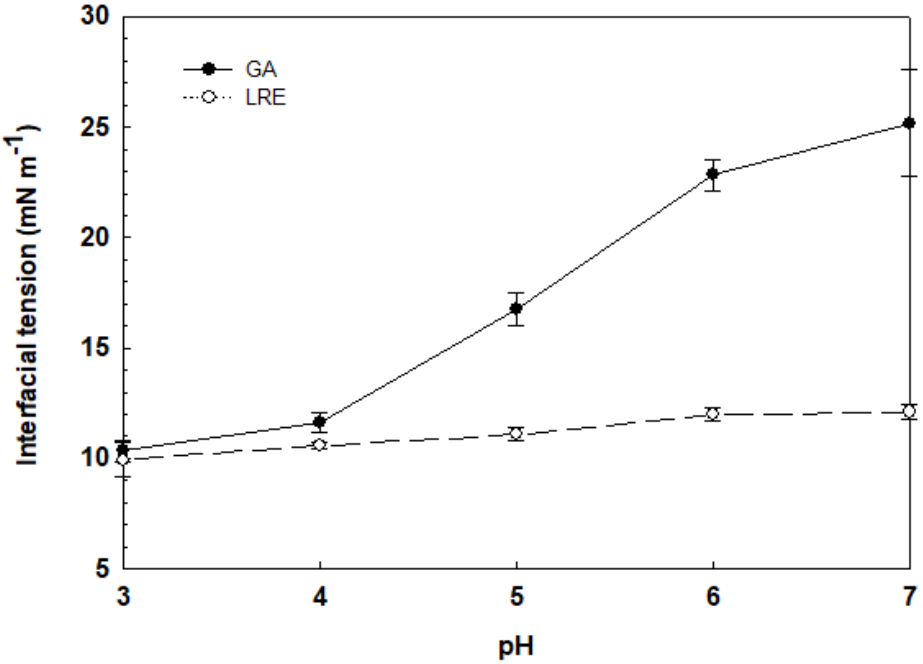


Figure 20. Effect of pH on the (a) viscosity and (b) interfacial tension of licorice root extract (LRE) and purified glycyrrhizin (GA), at constant glycyrrhizin concentration (0.1%, w/w). The interfacial tension between Milli-Q water and oil was 26.2 ± 0.7 mN m^{-1}, at 25 °C.

The differential organization of glycyrrhizin molecules in licorice root extract was also indicated by viscosity measurements. As shown in Figure 20b, the viscosity of glycyrrhizin solution gradually decreased by increasing the pH. Licorice root extract on the other hand did not show any change in viscosity over the entire range of pH studied, indicating that the characteristic network of glycyrrhizin molecules could not be formed.

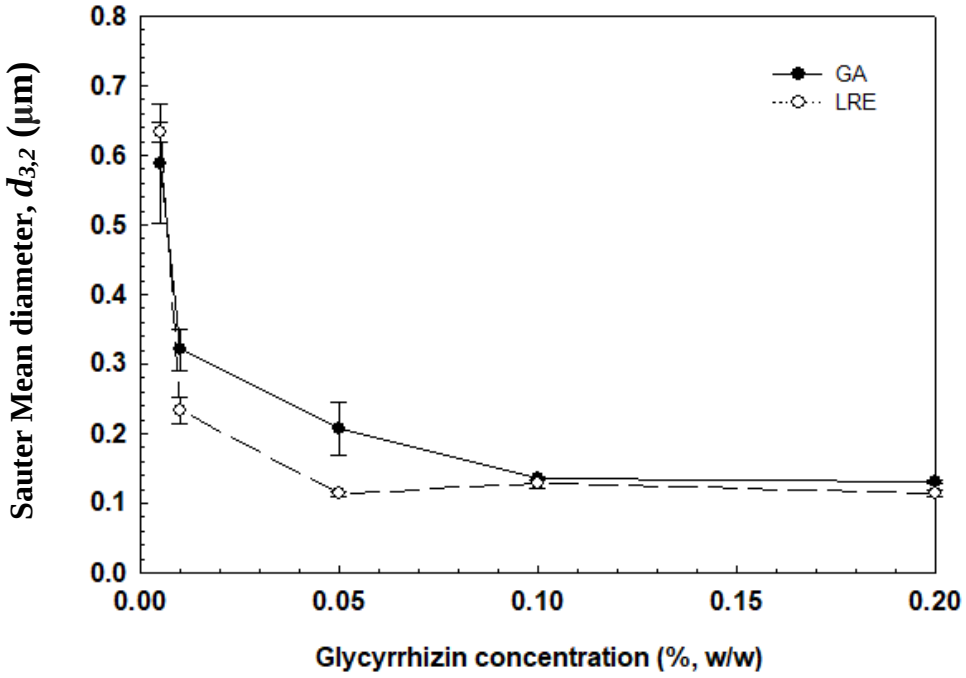
Evidences presented in this study indicate that the composition of licorice root extract specifically modulates the interfacial properties of saponins molecules, which can have a big impact during emulsion formation. We therefore, prepared emulsions, at constant concentrations of saponins, using licorice root extract or purified glycyrrhizin.

Emulsion formation

In this section, we evaluated the effect of emulsifier type on the droplet size and droplet size distribution of emulsions prepared at different glycyrrhizin and oil concentrations. As shown in Figure 21, the emulsions had similar droplet size at high concentration of glycyrrhizin (≥0.1, w/w). As we reduced this concentration, the emulsions started to show some signs of instability when prepared using purified glycyrrhizin. The results suggest that at low concentrations of glycyrrhizin, the interface was not completely covered leading to droplet coalescence. The presence of other surface-active compounds in licorice root extract however, could compensate to this reduction, maintaining a small droplet size.

Reichert et *al*., [40] previously showed that diminutive amounts of saponins in combination with proteins can form stable emulsions. The authors hypothesized the formation of saponin-protein complexes due to hydrophobic and/or electrostatic interactions. We therefore believe that the mutual presence of saponins and proteins in licorice root extract could have led to the formation

a)



b)

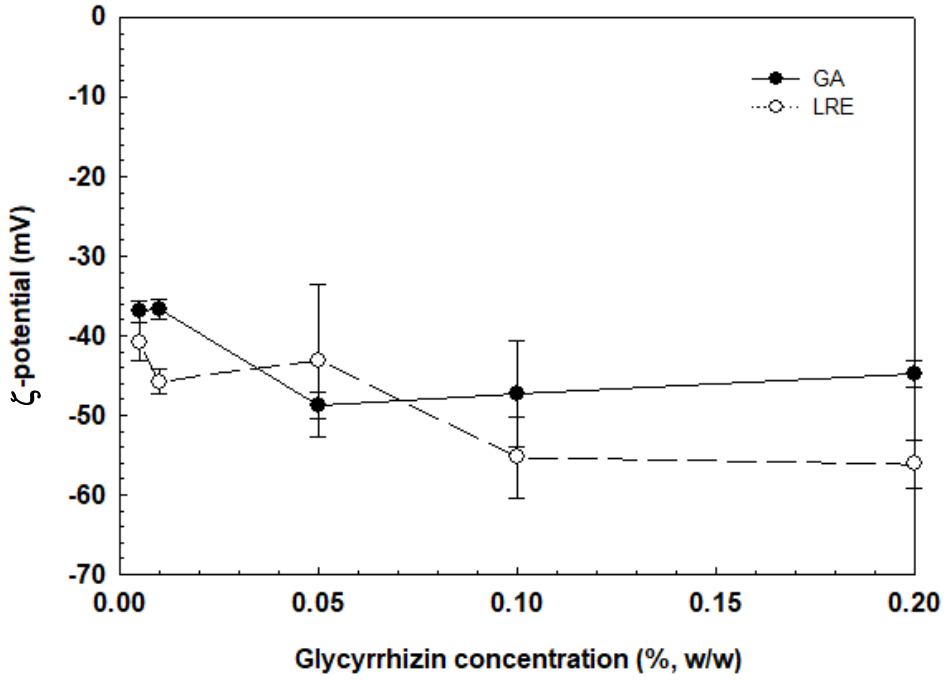


Figure 21. Effect of glycyrrhizin concentration in continuous phase on (a) volume mean diameter ($d_{4,3}$) and (b) the ς-potential of emulsions prepared using purified glycyrrhizin (GA) or licorice root extract (LRE) at pH 7.

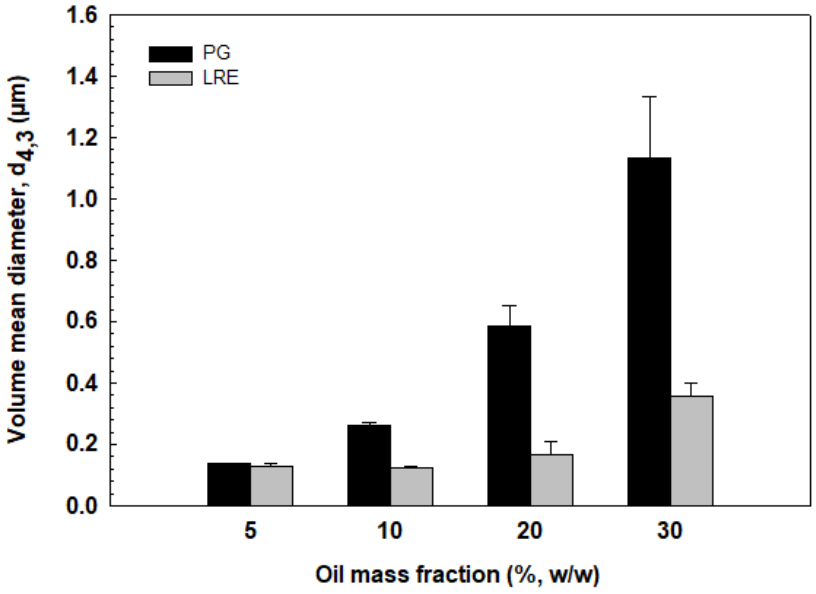
of highly surface-active complexes, with increased coverage to the droplets interface. This was also shown as we increased the concentration of oil in dispersed phase. Emulsions prepared using purified glycyrrhizin were prone to coalescence and increase in droplet size, while those prepared using licorice root extract were more stable (Figure 22). This is usually due to the insufficient presence of emulsifier molecules to cover all newly synthetized droplets, increasing the rate of coalescence [40]. We suggest therefore that emulsions prepared using licorice root extract were stabilized by the contribution of multiple compounds, particularly proteins and saponins, which resulted in improved droplet coverage and stability, as compared to those prepared using purified saponins.

Emulsions stability

In this section, we examined the effect of pH (3-9), NaCl concentration (0-400 mM) and temperature (40-120 C, 30 min) on the stability of emulsions prepared using licorice root extract of purified glycyrrhizin, at constant glycyrrhizin concentration (0.1%, w/w).

As described earlier (Figure 21), emulsions prepared using licorice root extract or purified glycyrrhizin had a narrow droplet size (< 200 nm) and negative ς-potential (~ -50 mV), when freshly prepared. Upon reducing the pH, the particle size of emulsions prepared using purified glycyrrhizin increased gradually, whereas that of emulsions prepared using licorice root extract did not change over the entire of pH studied (Figure 23). Glycyrrhizin have three carboxylic acids in its chemical structure, which dissociate at different pH. Among the three carboxylic acids, two dissociate at pKa 3.6, while the third carboxylic group of the aglycone dissociate at pKa 4.9 [80]. Lowering the pH of emulsions resulted therefore in glycyrrhizin molecules protonation, which resulted in less electrostatic repulsion between the droplets (Figure 23). In licorice root emulsions

a)



b)

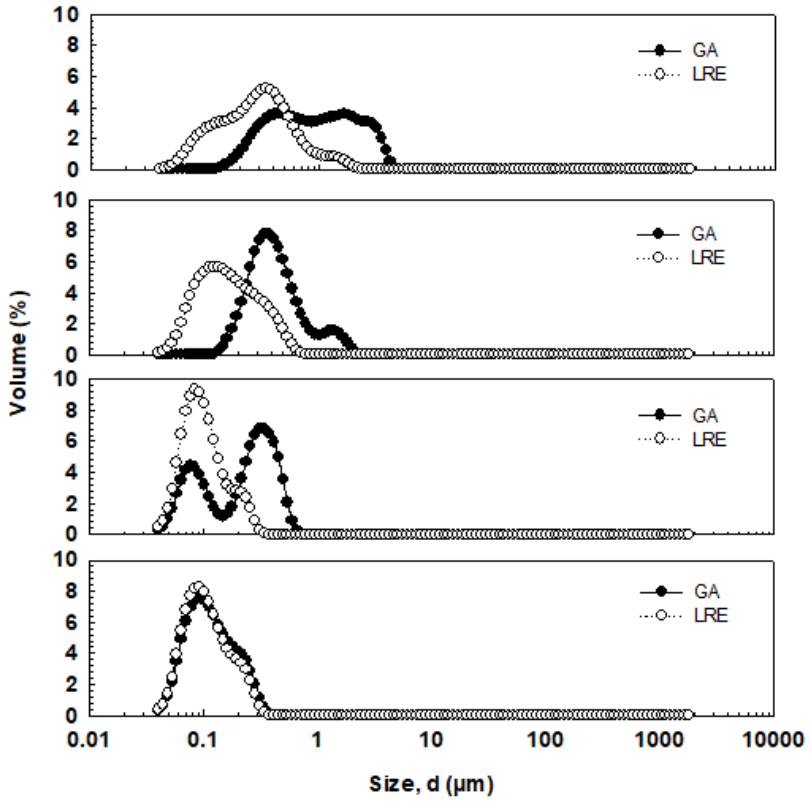


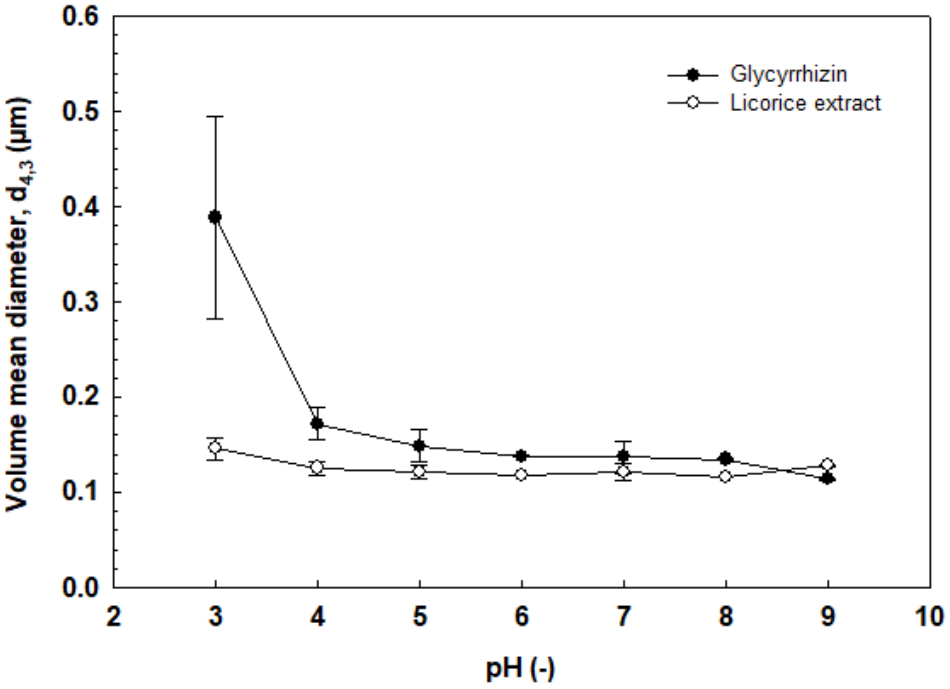
Figure 22. Effect of oil mass fraction on (a) the volume mean diameter ($d_{4,3}$) and (b) the droplet size distribution of soybean O/W emulsions prepared using purified glycyrrhizin (GA) or licorice root extract (LRE), at constant glycyrrhizin concentration (0.1%, w/w) and pH 7.

on the other hand, the presence of other surface-active compounds, particularly proteins, could have resulted in improved coverage of the droplets and therefore better stability at low pH (Figure 23).

Upon addition of salt, emulsions prepared using purified glycyrrhizin showed early signs of instability at low concentration of NaCl. There was a clear oil layer on the top of emulsions, indicating an extensive occurrence of coalescence. Licorice root extract-based emulsions on the other hand only resulted in aggregation instead of coalescence at high concentrations of salt ($\geq$ 200 mM). This is in good agreement with the previous observation about the effect of pH, suggesting a better droplet coverage of emulsions prepared using licorice root extract due to the presence of other surface-active compounds such as proteins.

The presence of proteins on droplet interface was also indicated by the effect of thermal treatment. As shown in figure 25, emulsions prepared using licorice root extract were highly unstable at elevated temperature, while those prepared using purified glycyrrhizin were less affected by increasing temperature. This behavior may be attributed to the presence of proteins in licorice root extract, leading to interface denaturation and consequently a lower surface activity.

a)



b)

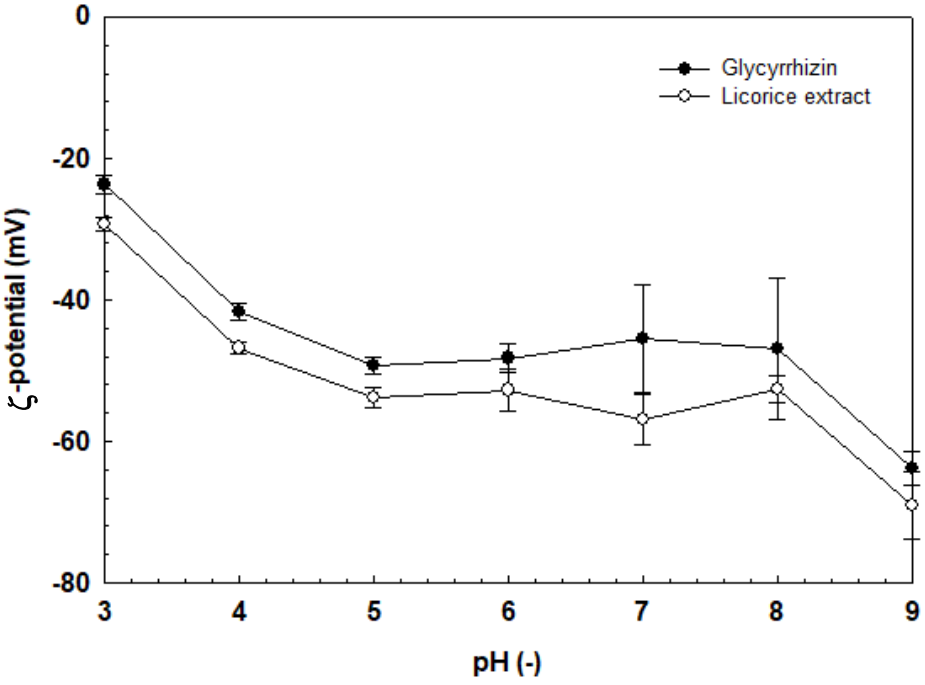
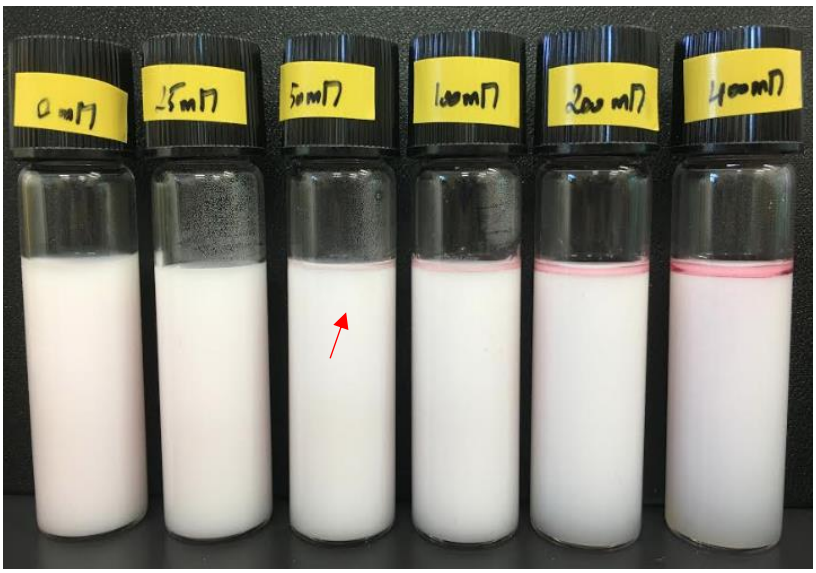


Figure 23. Effect of pH on (a) the volume mean diameter ($d_{4,3}$) and (b) the ς-potential of 5% (w/w) soybean O/W emulsions prepared using purified glycyrrhizin (GA) or licorice root extract (LRE), at constant glycyrrhizin concentration (0.1%, w/w) and pH 7.

Glycyrrhizin



Licorice root

Figure 24. Effect of NaCl concentration (0-400 mM) on the visual appearance of 5% (w/w) soybean O/W emulsions prepared using purified glycyrrhizin (GA) or licorice root extract (LRE), at constant glycyrrhizin concentration (0.1%, w/w) and pH7

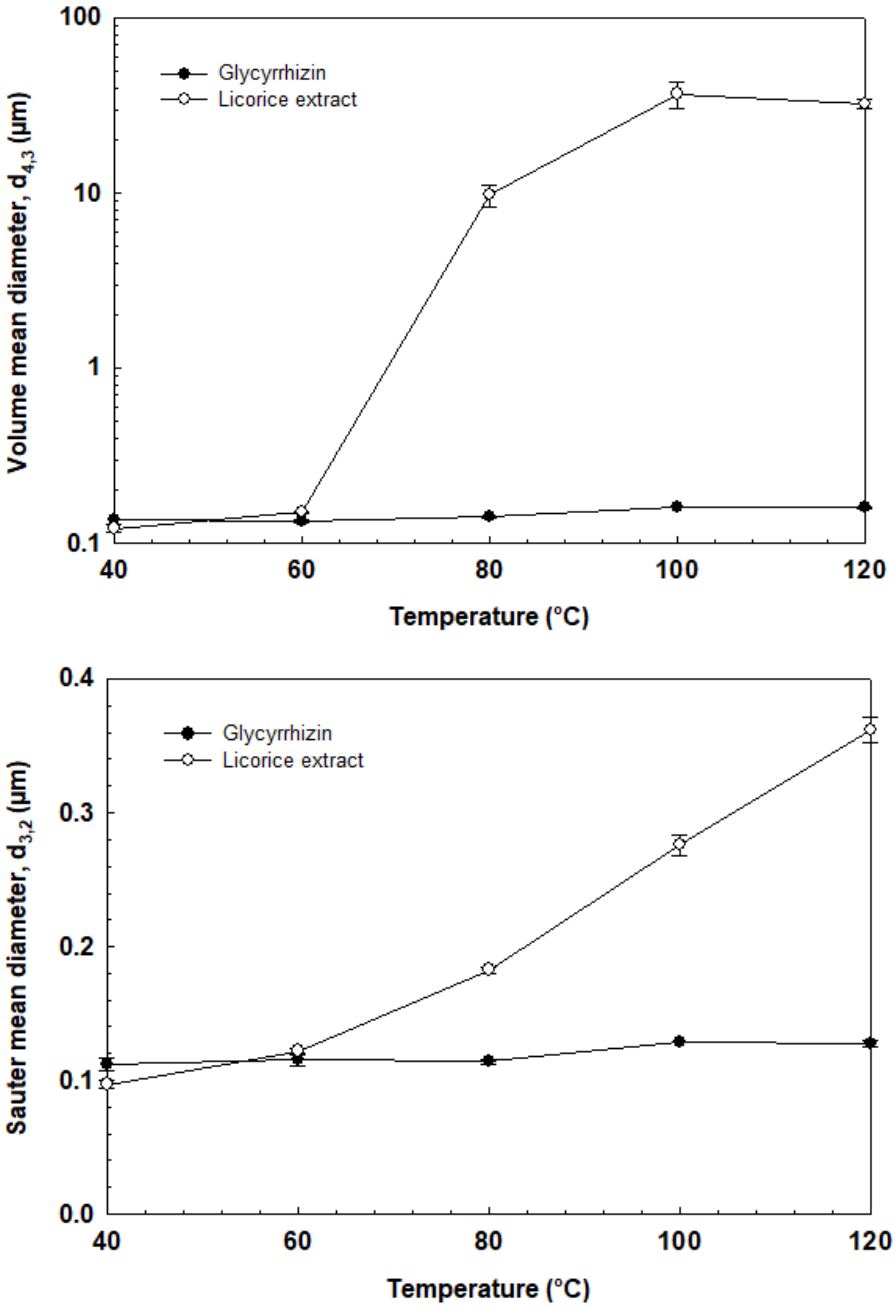


Figure 25. Effect of thermal treatment (40-120 °C) on (a) the volume mean diameter ($d_{4,3}$) and (b) the ς-potential of 5% (w/w) soybean O/W emulsions prepared using purified glycyrrhizin (GA) or licorice root extract (LRE), at constant glycyrrhizin concentration (0.1%, w/w) and pH 7.

Discussion

Labeling foods with tags such as ‘natural’ or ‘sustainable’ can have a big impact on product attractiveness and perceived quality [74]. State actors such as FDA and USDA usually control these tags and appoint certain criteria for their application. It is therefore important to explore innovative sources of food ingredients to make appropriate use of these labels.

Emulsifiers are one of the most utilized ingredients in food industry. They are ubiquitous in many food products, in which they provide multiple properties, such as good dispersibility, prolonged stability and/or improved bioavailability of other ingredients [7]. Many natural emulsifiers are currently available in the market. Among these, proteins, lecithin and polysaccharides are relevant [9]. The increasing demand of consumers towards ‘green label’ products have attracted however additional interest in identifying other sources of natural emulsifiers. Particularly, the use of crude plant emulsifying extracts, obtained via simple extraction/separation steps, can be of great interest. These extracts are a mixture of multiple compounds that can mutually enhance the emulsifying properties as compared to individual ingredients. They are also a mixture of other compounds that could negatively affect emulsions characteristics. In this study, we evaluated the interfacial and emulsifying properties of various aqueous-ethanolic extracts from argan by-products. Our aim was to produce stable emulsions using these extracts as sole emulsifiers. We also wanted to provide clear insights about their emulsifying mechanism in O/W emulsions.

Overall, we were able to produce stable emulsions using argan extracts as sole emulsifiers. The stabilization mechanism is believed to be exclusively electrostatic, likely due to the formation of biogenic complexes between proteins and saponins. The emulsions had similar physicochemical characteristics to those prepared using conventional emulsifiers and were stable up to two months

of storage at different temperatures. Nevertheless, for successful applications in the future, it would be important to extend this work by evaluating other properties such as the taste profile and the potential toxicity of the extracts.

Recent studies about the application of saponin-based emulsifiers in food emulsions have also pointed out to the necessity of extending their work by evaluating the taste characteristics of the final products [14, 50]. Nevertheless, they also claimed the good opportunity of applying these natural emulsifiers in food and/or beverage industries, such as Q-Naturale © 200, which is already commercialized and used as a food-grade emulsifier (Ingredion, 2016). Actually, many bioactive ingredients such as polyphenols and saponins have often a bitter taste, which may limit their application in foods [75]. Suppression of the bitter taste has therefore become a goal in food and nutraceuticals products development. One approach of suppressing bitterness currently involves the use of sweeteners (fructose, sucralose, stevia, etc.), flavors (vanillin, maltol) and/or bitter taste receptor blockers amino-acids [78]. Other approaches also include the use of cyclodextrins, lipids or emulsions [79]. Furthermore, in the case of emulsions, a recent sensory threshold study, showed that O/W emulsions exhibit bitterness-suppressing effects on KCl and/or caffeine without addition of any kind of sweeteners or flavors to the system [81]. Previous studies have also shown that emulsions characteristics such as fat/oil content, viscosity and droplet size affect the sensory characteristics of the final product. Particularly, smaller droplet sizes are more efficient in reducing the bitter taste intensity than larger droplet sizes [82, 83]. It is therefore agreed that the immobilization patterns and organization of the molecules in the emulsion system play a major role in bitterness perception of the final product. The short oral residence time and the low-viscosity of emulsions are also believed to reduce the sensory perception of bitterness [84, 85].

Nevertheless, we suggest that for a successful incorporation into foods products and/or beverages, new studies should also focus on de-bittering argan press-cake and associated extracts.

Additional concerns about the use of argan saponin-rich extracts in food emulsions should also consider the potential toxicity of the final product. Alaoui et *al*. [86] reported the acute and chronic toxicities (DL50) of a water extract of argan saponins: 1300 mg/kg and 200 mg/kg respectively. Bourhim et *al*. [87] reported that a saponin-rich extract from argan press-cake was not cytotoxic on B16 melanoma cells at all studied concentrations (0.01-2 mg/ml). However, no official agreement on the use of argan saponins or saponin-rich extracts in foods and/or cosmetics could be found to date. Quillaja saponins on the other hand are generally recognized as safe by FEMA (Flavor and Extract Manufacturers' Association) and accepted as natural flavoring substances for use in food and beverages [88]. Such reports are certainly important for an increased commercial value. Other studies should therefore focus also on evaluating other toxicity aspects of argan saponins. The information about their interaction in foods would be also of great interest to food industrials.

Cellulose from agricultural wastes has also attracted increasing interest in recent years [89]. Particularly, it can be used as a wall material for tableting pharmaceuticals, for fat replacement in foods and as an emulsion stabilizer [90]. The physicochemical properties of cellulose however are detrimental in determining its quality. Consequently, efforts must be done in order to obtain relatively low-cost cellulose with high quality. Hu et *al*. [91] developed a facile and efficient procedure to extract cellulose from argan press-cake. The obtained cellulose had a comparable molecular weight to bagasse cellulose but a lower degree of polymerization (DP) than cellulosic components obtained from other waste materials, such as corn stover. This is important for a successful application in food or pharmaceutical products, as low DP exhibits smaller particle size,

and therefore improved organoleptic and surface properties. In addition, argan press-cake cellulose prepared in this study had a low moisture content and relatively pure composition of glucose monomers. It was therefore qualified for use in food and pharmaceutical products without any further hydrolysis, as the case for cellulose extracted from other sources [91]. Different approaches for the extraction of cellulose was also investigated by Hattab [92]. The cellulose obtained by these methods however, particularly acid pulping and ethanol-water pulping, had lower quality and yield, and therefore deemed inferior. Efforts to improve the quality of extracted argan press-cake cellulose was also attempted by Benmassaoud et *al*. [93]. In this study, the authors could reduce the particle size of extracted cellulose by applying a supplementary step of acid hydrolysis. The obtained material was therefore qualified as nanocellulose (NC), with at least one dimension in the nanometer range. NC offers actually improved properties for the development of nanostructured-based materials. Furthermore, it can be an effective choice for extraction/clean up and preconcentration procedures [91]. Benmassaoud et *al*. [93] applied NC from argan press-cake as a sorbent material for the extraction of Sudan dyes in food samples. In one method they used NC as it is, along with the waste and eluents being separated by centrifugation. In another method, they modified the extracted NC by magnetic iron nanoparticles thus allowing magnetic separation. With both types of NC anyway, they could detect concentrations as low as 0.1 µg/L of Sudan dyes in barbecue and ketchup sauces [93]. In future, we could also think about the use of cellulosic materials from argan press-cake for the preparation of O/W emulsions.

The surge in nutrition-related chronic diseases has necessitated the need to search food ingredients with health-promoting properties. Bioactive peptides are oligomers that consist of 2–20 amino acids [94]. They demonstrate promising health-promoting properties including their antimicrobial roles, immunomodulatory functions, antioxidant, antihypertensive and anti-hyperglycemic

activities [95]. Bioactive peptides derive mostly from protein-rich raw materials following proteolysis, through enzymatic activities or fermentation or through chemical reactions [96]. They have been also extracted from different food by-products such as pumpkin and oilseed cakes [97, 98]. Hydrolysis of a protein fraction from argan press-cake was previously reported to yield a mixture of peptides of molecular weight below 20 000 Da. [4]. New areas of research should therefore explore the potential use of peptides from argan press-cake and argan press-cake extraction residue for food and pharmaceutical applications. There is also the need to validate the health-effects associated with the use of these peptides in human nutrition.

The looming food insecurity confronting several parts of the world has prompted increasing research interest on the utilization of plant-based by-products in staple food development. For example, the application of oily seeds press-cakes such as naked pumpkin seed (PuC), sunflower seed (SC), yellow linseed (LC), and walnut (WnC) in bakery products, especially bread, has been explored [97, 98, 99, 100]. In this particular study, 5% SC substitution with wheat flour resulted in breads that had appealing organoleptic perceptions to consumers and was not significantly different from the "control" sample. Although dough viscoelastic properties were impacted following PuC and SC substitution, the use of hydrocolloid replacement or hard-type wheat flour was recommended by the authors to address that challenge. The dietary influence of tocopherol and fiber-rich black currant seed press-cake on serum and stool tocopherol concentration was also investigated in a controlled human intervention study. The press-cake residue bread lead to significantly increased β-, γ-, δ- and total tocopherol intake, serum α-, β-, γ- and total tocopherol concentration, as well as fiber intake and urinary vitamin C concentration in comparison to the control bread [99]. Another study also evaluated the utilization of press-cakes from pumpkin seeds in biscuit formulation [100]. In a similar vein, the development of argan press cake residue-

enriched bread could be also investigated. Anecdotally, no documented information, whether successful on not, could be found on this regard. We could therefore suggest the use of argan press-cake extraction residue for such application. Here again, the presence of saponins and other secondary metabolites in the cake should be considered, as it may impart bitterness to the final product.

Finally, it can be concluded that argan by-products can have potential industrial applications in food industry. With the oil production which is estimated to quadruple by 2022, it is even more encouraging to investigate innovative approaches for efficient and sustainable valorization scenarios. Indeed, recent studies about argan by-products highlighted possible applications in food products and/or beverages. Thus, we think that sequential extraction methods should be adopted in the future to simultaneously obtain various bioactive and dietary compounds from these materials. First, surface-active compounds can be extracted for emulsion preparation, then cellulose, proteins or other major components can be obtained. Suitable storage conditions, innovative harvesting methods and adjusted oil production chain should be also considered in order to preserve specific natural compounds in the different parts of argan fruit, without affecting the productivity of the tree. Finally, for successful application in food and beverages industry, other parameters such as bitterness, the potential toxicity, the cost and the reliability of supply are important.

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Publications

Taarji, N., Cezar, A.R.D.S., Khalid, N., Gadhi, C., Hafidi, A., Kobayashi, I., Marcos, A.N., Isoda, H. & Nakajima, M (2018). Formulation and stabilization of oil-in-water nanoemulsions using a saponins-rich extract from argan oil press-cake. *Food Chemistry*, 246, 457-463.

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Appendix

Table 1. Estimative generated quantities (tons) of argan oil extraction by-products in 2014. Calculations were made based on global argan oil market in 2014* [101], average oil extraction yield by mechanical press (45% from kernels) [102] and average composition of dry argan fruit [103].

Product/by-product	**Dry fruit average composition (%)**	**Estimative quantity (tons)**
Argan kernels	6	-
Argan oil	2.7	4835.5*
Argan oil press-cake	3.3	5910
Argan pulp	45	80592
Argan nut-shell	49	87755
Total	97.3	174257

Table 2. Overview of the major organic (%, dw) and mineral (ppm, dw) composition of argan by-products [103].

	Argan press-cake	**Argan pulp**	**Argan nut-shell**
Proteins	48.4	8.7	3.3
Crude fat (Ether extract)	6.9	6.6	2.7
Non-cellulosic carbohydrates	18.7	42.9	19.5
Fibers	20.7	34.5	73.6
Ash	5.3	-	-
Ca	6900	4800	900
P	6400	0	0
Na	400	2400	600
K	10400	23200	4500
Mg	3300	700	200
Cu	13.7	2.3	1.7
Zn	71.7	7.5	6.1
Mn	59.7	7	7.8
Fe	-	11.2	143.3

Table 3. Overview of the minor chemical composition (ppm, dw) of argan by-products. NF: Not Found

Chemical class	Major compounds	Extraction solvent (v:v)	Content (ppm)	Reference
Argan press-cake				
Saponins (*ppm of extract)	Arganins A-F, Mi-saponin A	Ethanol:Water (80:20)	5000	[26]
	Arganins A-F, Mi-saponin A, glucuronic-oleanane type saponins	Ethanol:Water (50:50)	22400	[25]
		Ethanol	2800	
	Unidentified (total saponins)	Water	26000*	[46]
		Ethanol:Water (20:80)	35000*	
		Ethanol:Water (50:50)	41000*	
		Ethanol:Water (80:20)	36000*	
		Ethanol 99.5%	12000*	
Polyphenols	Catechin, epicatechin, protocatechic acid	Methanol:Water (80:20)	25.4 - 286.6	[104]
	Epicatechin, unknown (MW = 457.3)	Acetone:Water:Formic acid (70:29.5:0.5)	7300 - 7900	[105]

Argan pulp				
Polyphenols	Protocatechic acid, epicatechin, hyperoside	Methanol:Water (80:20)	NF	[106]
	Catechin, procyanidin, glycosylates	Methanol	15400	[107]
	Isoquercetin, hyperoside	Acetone:Water:Formic acid (70:29.5:0.5)	75800	[105]
Triterpenoids (*ppm of unsaponifiable fraction)	Erythrodiol, lupeol, b-amyrin	NF	240000*	[103]
Essential oil	Ursolic acid, oleanolic acid	Ethanol	6300	[108]
	Camphor	Water (hydrodistillation)	600	[109]
	Resorcinol	Ethyl ether	NF	[110]
Argan nut-shell				
Saponins	Neutral-oleanane type saponins	Methanol	11250	[111]
Polyphenols	Epicatechin, isoquercetin	Acetone:Water:Formic acid (70:29.5:0.5)	4400	[105]

Table 4. Overview of the biological activities of natural extracts obtained from argan by-products.

Biological role	Extract type	Bioactive compounds/mechanism	Reference
Free radical scavenging activity	Pulp, shell and press-cake acetone:water:formic acid (70:29.5:0.5) extracts	Polyphenols and Maillard reaction products formed during the roasting step of kernels (roasted argan press-cake)	[105]
Depigmenting effect	Press-cake ethanol:water (70:30) extract	Saponins, phenolic acid derivatives and flavonoids by inhibiting the biosynthesis of melanin	[87]
Anti-inflammatory activity	Press-cake	Saponins by their action on leucotriens in the metabolic pathway of arachidonic acid, through free radicals scavenging	[112]
Protection against oxidative haemolysis	Press-cake aqueous-ethanolic extract	Saponins by providing free radicals scavenging activity	[112]
Anticancer effect (Human prostate cancer)	Press-cake methanolic extract	Saponins	[113]
Anticancer effect (Human breast cancer)	Ground fruits (flesh) ethyl acetate extract	Anthocyanins	[114]
Insulin-sensitizing effect	Press-cake methanolic extracts	Saponins by affecting insulin-response mediation key enzymes	[115]

Table 5. Patent grants related to the application of argan by-products in dermocosmetic formulations.

Patent grant	**Grant Date**	**Title**	**Summary**	**Inventors**	**Assignee**
US7871766	2011	Cosmetic and/or dermopharmaceutical preparations containing native proteins from the plant *Argania spinosa*	Skin care preparations containing native proteins from argan oil press-cake	Pauly, G., Henry, F., Moser, P. & Charrouf, Z.	BASF
EP1711194	2009	A plant extract and its pharmaceutical and cosmetic use	Use of a triterpenoid fraction from argan pulp for the production of skin care formulations	Henry, F., Danoux, L. Pauly, G. & Charrouf, Z.	BASF
US20060083794	2006	Use of an extract from the plant *Argania spinosa*	Use of extracts from *Argania spinosa* for the production of anti-acne and/or anti-seborrhea preparations, with anti-5-α-reductase activity	Henry, H., Danoux, L., Pauly, G. & Charrouf, Z.	BASF

EP1276460	2006	Use of preparations containing an extract of the plant *Argania spinosa* in cosmetic care products for hair and skin	Cosmetic preparations containing a saponins-rich extract from argan oil press-cake	Henry, F., Pauly, G., Moser, P. & Charrouf, Z.	BASF
US7105184	2006	Cosmetic and/or dermopharmaceutical preparations containing leaf extracts of the plant *Argania spinosa*	Cosmetic preparations comprising a mixture of flavonoids, saponosides and sterols, extracted from *Argania spinosa* leaves	Pauly, G., Henry, F., Danoux, L. & Charrouf, Z.	BASF
INPI2553788	1985	Lipid extract of argan fruit, process of preparation and application in cosmetology	New process for the preparation of a stable lipid extract of argan fruit and its application in skin care formulations	Hatinguais, P., Therese, M. & Belle, T.R.	Pierre Fabre

US: United states patent; EP: European patent; INPI: National Institute of Industrial Property.