

## Article

# Emulsion Formation and Stabilizing Properties of Olive Oil Cake Crude Extracts

Firdaous Fainassi <sup>1</sup>, Noamane Taarji <sup>1,2,\*</sup>, Fatiha Benkhalti <sup>3</sup>, Abdellatif Hafidi <sup>1</sup>, Marcos A. Neves <sup>4</sup> , Hiroko Isoda <sup>4</sup> and Mitsutoshi Nakajima <sup>4</sup>

<sup>1</sup> Department of Biology, Faculty of Sciences-Semlalia, Cadi Ayyad University, Marrakech 40001, Morocco; fainassi.f@gmail.com (F.F.); a.hafidi@uca.ac.ma (A.H.)

<sup>2</sup> Food and Medicinal Resource Engineering Open Innovation Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-0821, Japan

<sup>3</sup> Sustainable Development and Health Research Laboratory, Cadi Ayyad University, Marrakech 40000, Morocco; benkhalti.f@gmail.com

<sup>4</sup> Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan; marcos.neves.ga@u.tsukuba.ac.jp (M.A.N.); isoda.hiroko.ga@u.tsukuba.ac.jp (H.I.); nakajima.m.fu@u.tsukuba.ac.jp (M.N.)

\* Correspondence: taarji.noamane@aist.go.jp

**Abstract:** The surface-active and emulsifying properties of crude aqueous ethanolic extracts from untreated olive oil cake (OOC) were investigated. OOC extracts contained important concentrations of surface-active components including proteins, saponins and polyphenols (1.2–2.8%, 7.8–9.5% and 0.7–4.5% (*w/w*), respectively) and reduced the interfacial tension by up to 46% ( $14.0 \pm 0.2 \text{ mN m}^{-1}$ ) at the oil–water interface. The emulsifying ability of OOC extracts was not correlated, however, with their interfacial activity or surface-active composition. Eighty percent aqueous ethanol extract produced the most stable oil-in-water (O/W) emulsions by high-pressure homogenization. The emulsions had average volume mean droplet diameters of approximately  $0.4 \mu\text{m}$  and negative  $\zeta$ -potentials of about  $-45 \text{ mV}$ , and were stable for up to 1 month of storage at 5, 25 and  $50 \text{ }^\circ\text{C}$ . They were sensitive, however, to acidic pH conditions ( $<5$ ) and NaCl addition ( $\geq 25 \text{ mM}$ ), indicating that the main stabilization mechanism is electrostatic due to the presence of surface-active compounds with ionizable groups, such as saponins.

**Keywords:** olive oil extraction by-product; interfacial tension; oil-in-water emulsion; protein; saponin; polyphenol



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## 1. Introduction

Emulsifiers are one of the most important ingredients in food industry. They are utilized as stabilizers and/or encapsulants in many food products and beverages in which they provide multiple properties such as good dispersibility, prolonged stability and improved bioavailability of other ingredients [1]. Many emulsifiers used nowadays derive from the same chemical and/or enzymatic reactions that were primarily designed in the past for mass and economic production of these substances [1,2]. The current trend towards natural and sustainable production of food emulsions, however, has led manufacturers to find new natural alternatives to synthetic emulsifiers [2]. Plant-derived compounds such as proteins, saponins, polysaccharides and phospholipids have shown promising emulsifying properties in various food applications [2,3]. They can provide similar stabilizing properties as synthetic emulsifiers, but they are also criticized because of their extensive preparation/manufacturing procedures [4–6].

A new trend in the utilization of food emulsifiers suggest the use of crude plant surface-active extracts, obtained via simple extraction/fractionation steps, to produce stable emulsions. Agro-industrial by-products contain various surface-active substances with strong emulsifying properties and can be utilized, therefore, as a source of these ingredients.

Sugar beet, Panax ginseng, oat bran, argan and bagasse by-products extracts have been successfully used, for example, to prepare stable oil-in-water (O/W) emulsions [7–12]. The active ingredients in these “crude” emulsifiers differ from one extract to another but they are, generally, attributed to a mixture of various surface-active components synergizing for an enhanced effect.

Olive oil processing by-products come in different forms and chemical profiles, depending on the olive ripening stage and the oil extraction method. In the traditional three-phase decanter systems, the solid waste is dry and contains significant contents of residual oil (2–4%, *w/w*), while in the case of two-phase decanter systems, a high-humidity (68–71%, *w/w*) semi-solid waste “Alperujo”, here referred to as olive oil cake “OOC”, is generated [13]. The major components found in olive solid by-products are polysaccharides (lignin 26–30%, hemicellulose 7–9% and cellulose 7–9%, *w/w*), proteins 5–7% (*w/w*), fatty acids 5–8% (*w/w*) and polyphenols (oleuropein, hydroxytyrosol and tyrosol) [14]. Such chemical composition makes solid olive processing wastes stand out as a promissory source of bioactive and nutritional compounds, especially in response to the rising trend of finding natural alternatives to synthetic ingredients.

Despite their abundance and valuable composition, studies investigating the emulsifying capacities of olive solid wastes are scarce. Filotheou et al. [15] used alcohol-insoluble olive processing wastes as a source of surface-active components to prepare acidic and non-acidic model food emulsions. The emulsifying efficiency was mainly attributed to the synergy between the surface-active macromolecular components and smaller surfactants. Koliastasi et al. [16,17] investigated the impact of partial and total composting on the surface-active composition and concluded that such treatments can produce emulsifiers with enhanced performances as compared to the extracts of non-composted materials. However, none of the previous studies have considered the use of crude extracts involving basic solid–liquid extraction from raw untreated material as emulsifiers. In this study, we evaluated the surface-active and emulsifying properties of various aqueous ethanolic extracts from olive oil extraction by-products. Our aim is to produce stable oil-in-water (O/W) emulsions using these extracts as sole emulsifiers.

## 2. Materials and Methods

### 2.1. Materials

Olive oil cakes (Picholine marocaine cultivar) obtained from a two-phase decanter system were provided by an industrial olive oil extraction unit in the region of Marrakech, Morocco. All other products were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless stated elsewhere.

### 2.2. Sample Preparation and Extraction Yields

The samples (100 g) were added to fresh mixtures (300 mL) of distilled water and ethanol and stirred for 3 h at room temperature. The suspensions were then centrifuged at  $2000 \times g$  for 30 min, filtered using Whatman filter paper type 111A and vacuum evaporated at 40 °C and 16 hPa (Eyela EVP-1100, Shanghai Co., Ltd., Shanghai, China) to remove solvent. The dried extracts were finally dispersed in distilled water, re-filtered using 0.45- $\mu\text{m}$  hydrophobic PTFE membrane filters (RephiLe Bioscience, Co., Ltd., Shuangbai, China) and freeze-dried at  $-80$  °C and 4 Pa (Eyela FDH-2110, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to remove water. The yields of extraction (EY) were calculated as follows:

$$\text{EY (\%, wet basis)} = W_1/W_0 \times 100 \quad (1)$$

where  $W_1$  represents the weight of the extract after the freeze-drying and  $W_0$  represents the weight of the fresh olive oil cake.

### 2.3. Physicochemical Characterization

Protein content was determined using a total nitrogen analyzer (UNICUBE, Elementer Ltd., Yokohama, Japan) with a nitrogen-to-protein conversion factor of 6.25. Saponins

content was determined following the method of Zhaobao et al. [18], using oleanolic acid as standard (0–192 µg). Phenolic compounds content was determined following the Folin–Ciocalteu method [19] using gallic acid as standard (0.1–0.5 g L<sup>-1</sup>). Interfacial tension was measured using the pendant drop method (PD-W, Kyowa Interface Science Co., Ltd., Saitama, Japan) at the soybean oil/water interface [10]. Particle size was determined using a dynamic light scattering particle size analyzer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK).

#### 2.4. Emulsion Preparation

Coarse emulsions were prepared by homogenizing 0.1–5% (*w/w*) extract and 2.5–10% (*w/w*) soybean oil at 10,000 rpm for 5 min (Polytron PT-3000, Kinematica Inc., Luzern, Switzerland). The coarse 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.

#### 2.5. Emulsion Stability Evaluation

##### 2.5.1. Effect of pH

Emulsions were diluted with phosphate buffer solutions (10 mM) to obtain a final oil content of 2.5% (*w/w*). The pH was then readjusted to appropriate level (2–9), using 1 M HCl or NaOH, and the samples were stored for 24 h at 5 °C before analysis [9].

##### 2.5.2. Effect of Ionic Strength

Emulsions were diluted with phosphate buffer solutions (10 mM) of appropriate NaCl concentration (50–800 mM) to obtain a final oil content of 2.5% (*w/w*). The pH was then readjusted to 7 and the emulsions were stored at 5 °C until analysis [10].

##### 2.5.3. Effect of Long-Term Storage

Sodium azide was added to the freshly prepared emulsions at a final concentration of 0.02% (*w/w*). The emulsions were then incubated for 30 days at 5, 25 or 50 °C prior to analysis.

#### 2.6. Droplet Characterization

Volume mean droplet diameter ( $d_{4,3}$ ) was measured using a static laser diffraction particle size analyzer (LS 13 320, Beckman Coulter, Brea, USA). Droplet charge was measured using a  $\zeta$ -potential analyzer (Zetasizer, Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). In the case of  $\zeta$ -potential measurements, the emulsions were diluted (1/100) with deionized water or phosphate buffer solutions (10 mM) of appropriate pH and salt concentration, prior to analysis, to prevent multiple scattering effects. When using the Beckman Coulter, the emulsions were directly injected into the measurement module without dilution.

#### 2.7. Data Analysis

All measurements were performed in duplicate using two independently prepared samples. The reported values represent means and standard deviations. The measurements were subjected to analysis of variance (ANOVA) using the “Tukey” test to assess significant differences among variables at 95% confidence level using Excel (Microsoft, Redmond, WA, USA).

### 3. Results and Discussion

#### 3.1. Physicochemical Properties of OOC Extracts

##### 3.1.1. Surface-Active Composition

Proteins and saponins are known to have surface-active properties due to the presence of hydrophilic and hydrophobic moieties in their chemical structures [20,21]. Polyphenols are also known to exhibit surface-active properties, contributing to the formation and

stabilization of O/W emulsions [22]. Moreover, they can act as antioxidants in many food products and beverages, providing additional properties such as chemical stability and antioxidant activity to the prepared emulsions [23]. In this section, we evaluated the effect of extraction solvent on the concentration of proteins, saponins and phenolic compounds in OOC extracts. As shown in Table 1, increasing the concentration of ethanol did not have a strong effect on protein and saponin contents; even in the absolute ethanol extract where it was expected that the concentration of proteins would be reduced appreciably due to protein precipitation, the concentration was close to that obtained by the water extract. Phenolic compound concentration, on the other hand, was appreciably reduced in the absolute ethanol extract (Table 1).

**Table 1.** Extraction yields (EY) and content in surface-active components (% *w/w*) of olive oil cake (OOC) extracts prepared using various aqueous-ethanolic solvent mixtures. Means in the same line followed by the same letter are not significantly different ( $\alpha = 0.05$ ).

	OOC 0%	OOC 20%	OOC 40%	OOC 60%	OOC 80%	OOC 100%
EY % ( <i>w/w</i> )	10.2 ± 0.9 <sup>de</sup>	11.6 ± 1.1 <sup>cd</sup>	9.6 ± 0.9 <sup>e</sup>	12.0 ± 1.2 <sup>c</sup>	16.6 ± 1.9 <sup>b</sup>	22.4 ± 2.3 <sup>a</sup>
Proteins	1.79 ± 0.14 <sup>c</sup>	2.86 ± 0.12 <sup>a</sup>	2.27 ± 0.19 <sup>b</sup>	2.23 ± 0.14 <sup>b</sup>	2.19 ± 0.14 <sup>b</sup>	1.20 ± 0.17 <sup>d</sup>
Saponins	7.80 ± 0.38 <sup>c</sup>	8.07 ± 0.58 <sup>bc</sup>	8.71 ± 0.67 <sup>ab</sup>	9.01 ± 1.07 <sup>ab</sup>	8.38 ± 0.86 <sup>bc</sup>	9.29 ± 0.68 <sup>a</sup>
Polyphenols	4.58 ± 0.26 <sup>a</sup>	4.20 ± 0.22 <sup>a</sup>	3.23 ± 0.14 <sup>b</sup>	4.27 ± 0.23 <sup>a</sup>	3.49 ± 0.11 <sup>b</sup>	0.72 ± 0.04 <sup>c</sup>

Note: The shared letter(s) in the respective column indicate(s) no significant difference ( $p < 0.05$ ).

### 3.1.2. Particle Size Distribution

Proteins, saponins and phenolic compounds can interact by the effect of low molecular forces such as hydrogen bonds, electrostatic and hydrophobic interactions, in the bulk water phase, prior to adsorption or directly at the oil/water interface following adsorption of the slowly diffusing component on the pre-adsorbed layer of the other component [24–27]. To evaluate the structural organization of surface-active components in our extracts, and to gain better insight about their adsorption mechanisms, we evaluated the particle size and particle size distribution of aqueous phases containing OOC extracts. As shown in Figure 1, OOC extracts aqueous solutions (1%, *w/w*) had broad particle size distributions with average particle sizes of more than 100 nm. Moreover, except for 40% (*w/w*) aqueous ethanol extract, all samples had no particles smaller than 50 nm, indicating the presence of relatively large structures. Small molecule surfactants, such as saponins, form micelles of approximately 10 nm [28]. We suggest, therefore, that OOC extracts contain relatively large aggregates that result from the intermolecular interactions between various surface-active components, including proteins, saponins and phenolic compounds.

### 3.1.3. Interfacial Activity

Interfacial tension of emulsifiers plays an important role in determining their ability to form and stabilize emulsions. Usually, the lower the interfacial tension is, the greater the emulsifying properties are [29]. We measured, therefore, the interfacial tension of OOC extracts at the oil/water interface and compared their surface-active properties to commonly used emulsifiers. As shown in Figure 2, all extracts reduced their interfacial tension at the soybean oil/water interface, independently of extract composition. Extract 40% (*w/w*) aqueous-ethanol, for example, reduced its interfacial tension to approximately 13 mN m<sup>-1</sup>, while 80% (*w/w*) aqueous ethanolic extract was less efficient at about 16 mN m<sup>-1</sup>. These values are lower than the ones recorded from either non-composted or composted olive waste derived extracts [15,17]. They are in the same range, however, of previously evaluated extracts from sugar beet, bagasse, liquorice and argan press-cake extracts as well as purified natural emulsifiers, including proteins, saponins and phospholipids [7,10,12,30–33].

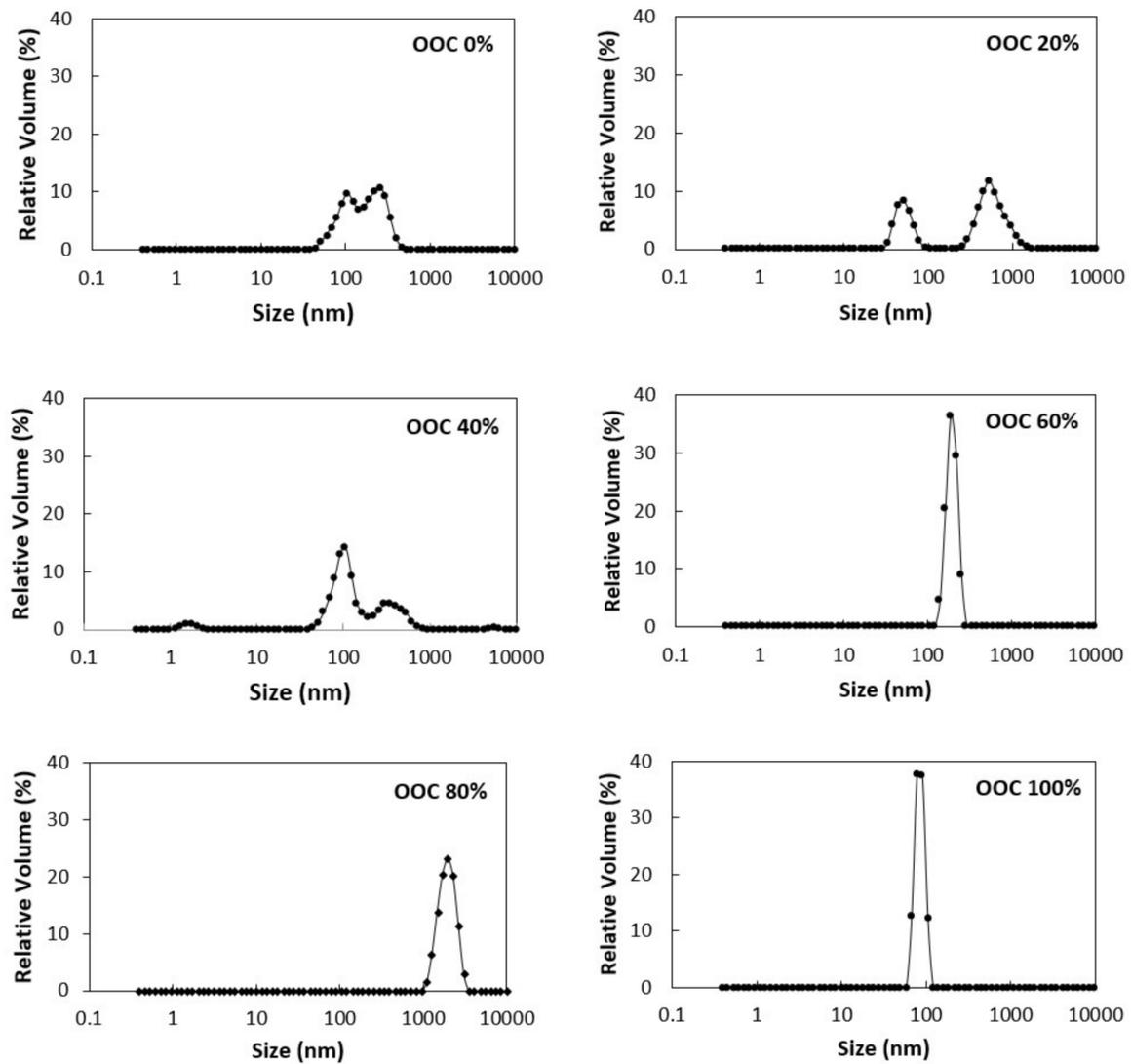


Figure 1. Particle size distribution of aqueous phases containing 1% (*w/w*) OOC extracts.

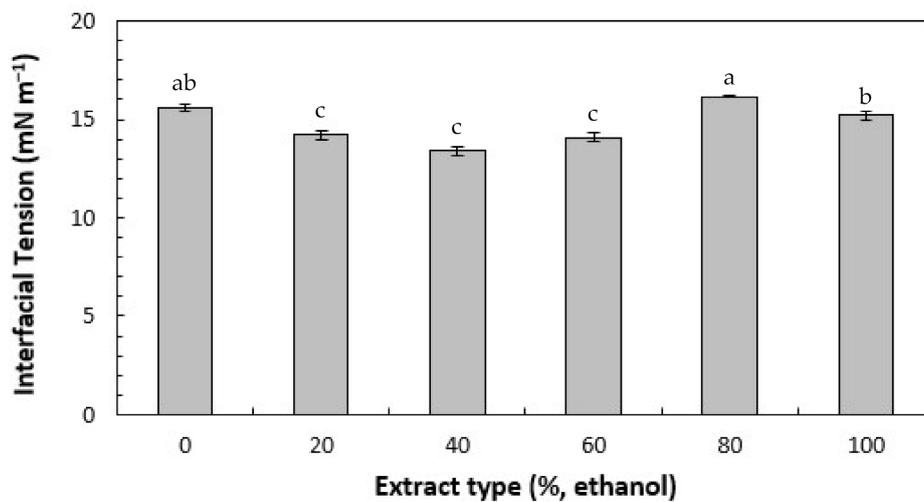
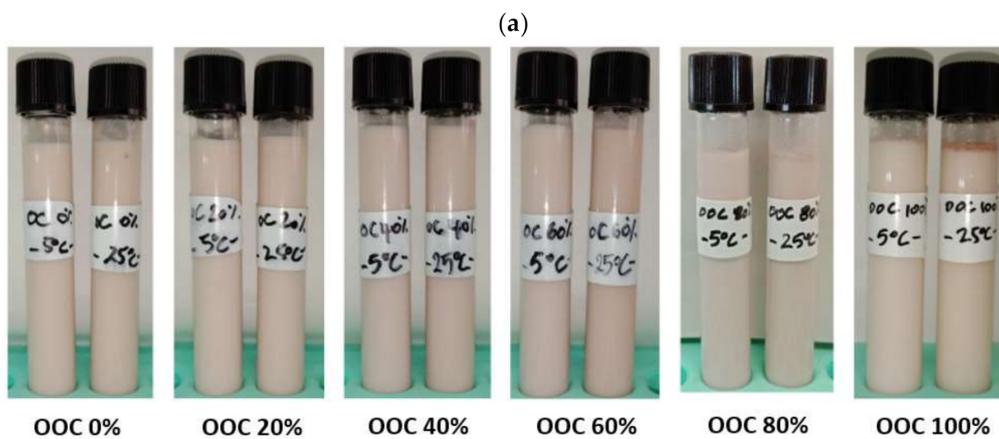
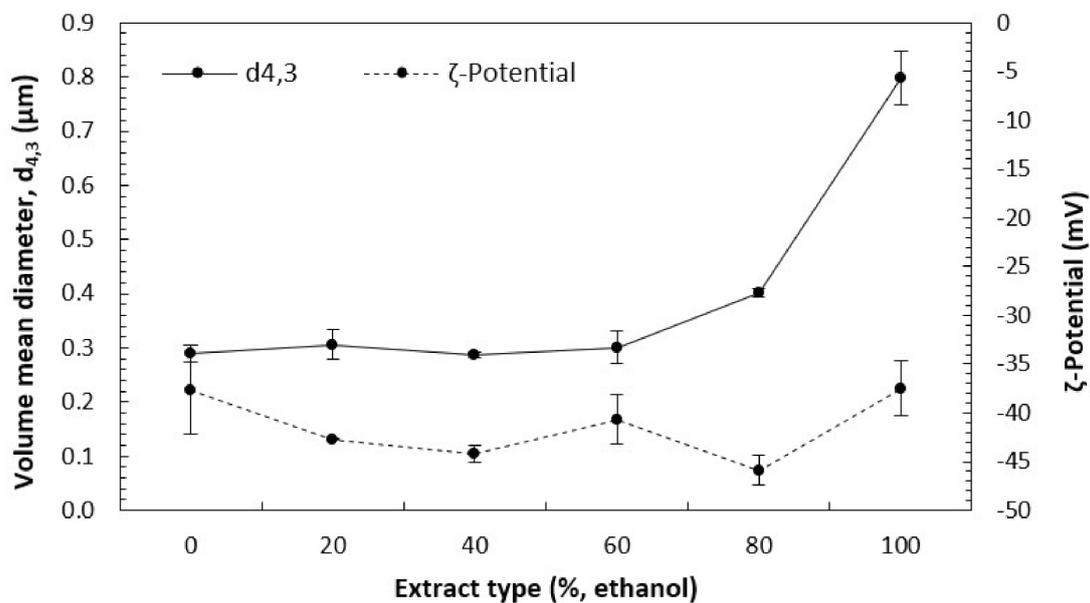


Figure 2. Interfacial tension of aqueous phases containing crude OOC extracts (1%, *w/w*). The interfacial tension between soybean oil and Milli-Q water was approximately 26 mN m<sup>-1</sup>. The shared letter(s) indicate(s) no significant difference ( $p < 0.05$ ).

### 3.2. Effect of Extract Type on the Formation Characteristics of O/W Emulsion

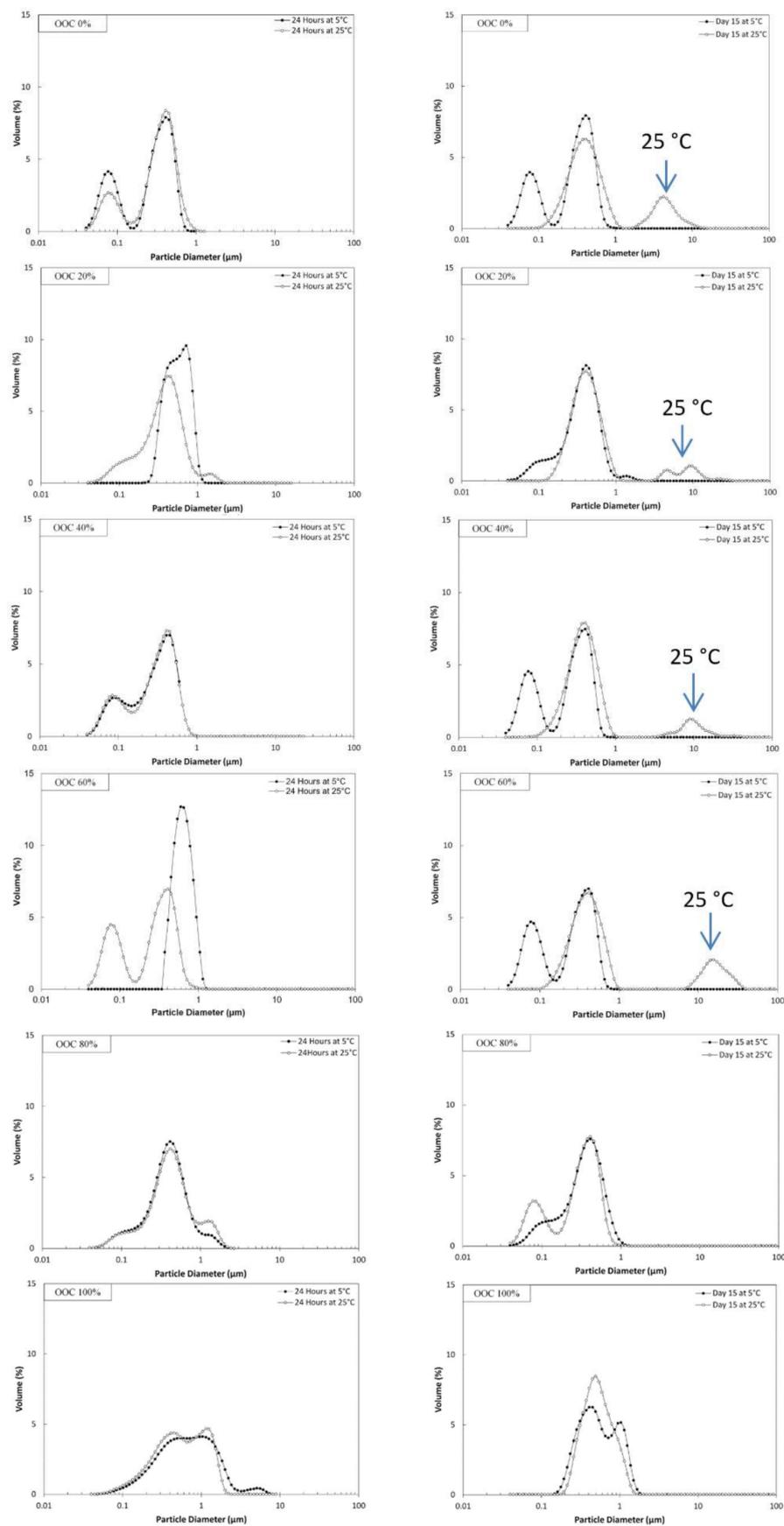
#### 3.2.1. Droplet Size

In order to evaluate the emulsifying performance of OOC extracts, O/W emulsions were prepared using aqueous solutions (1%, *w/w*) of each extract and 5% (*w/w*) soybean oil at standard homogenization conditions (100 MPa, four passes). Except for emulsions prepared using absolute ethanol extract, which showed immediate oiling-off after homogenization, all emulsions prepared using OOC extracts were successfully produced with average droplet sizes ( $d_{4,3}$ ) of 0.3–0.4  $\mu\text{m}$  (Figure 3a). When stored at 5 °C, the emulsions showed no evidence of droplet growth or phase separation for up to 15 days with a main peak around 0.4–0.6  $\mu\text{m}$  (Figure 4). At 25 °C, the peak around 0.4  $\mu\text{m}$  was maintained but for the emulsions prepared using 0, 20, 40 and 60% (*w/w*) ethanol-to-water-ratios, droplet growth occurred with the emergence of lesser peaks around 4.5–15  $\mu\text{m}$ . Interestingly, emulsions prepared using 80% (*w/w*) aqueous ethanolic extract were stable at all studied temperatures despite their close surface-active composition and interfacial activity to other extracts. With a main peak around 0.4  $\mu\text{m}$  and a lesser peak around 0.08  $\mu\text{m}$  (Figure 4), OOC 80% appears to be a better emulsifier and was used, therefore, for further experiments.



(b)

**Figure 3.** (a) Volume mean droplet diameter ( $d_{4,3}$ ) and  $\zeta$ -potential of soybean oil-in-water (O/W) emulsions prepared using OOC aqueous ethanolic extracts, by high-pressure homogenization (100 MPa, 4 passes). (b) Visual appearance of OOC extract-stabilized emulsions after 15 days of storage at 5 °C or 25 °C.



**Figure 4.** Droplet size distributions of emulsions prepared using 1% (w/w) OOC extracts and 5% (w/w) soybean oil, by high-pressure homogenization (100 MPa, 4 passes).

Previous studies have evaluated the emulsification capacity of extracts obtained from composted olive oil waste materials [16,17]. The studied emulsifiers produced larger droplets with a main peak around 2 to 3  $\mu\text{m}$  and a relatively constant droplet size distribution for 15 days of storage. The emulsifiers studied in this work encourage, therefore, the use of crude extracts, instead of time-consuming composting materials to produce stable O/W emulsions with smaller droplet sizes and a similar period of storage.

### 3.2.2. Droplet Surface Charge

The surface charge of emulsion droplets plays an important role in the formation and stabilization of emulsions. In general, a higher  $\zeta$ -potential means that the emulsifier layer yields more repulsive forces between emulsion droplets, thus preventing their coalescence. A neutral  $\zeta$ -potential, on the other hand, means that the emulsifier covered interfaces are more likely to destabilize, resulting in the formation of larger particles [33]. As shown in Figure 3a, all emulsions prepared using OOC extracts displayed a strong negative  $\zeta$ -potential, independently of their stability characteristics. The 80% (*w/w*) aqueous ethanol extract, for example, provided a  $\zeta$ -potential of  $-45.9$  mV, in agreement with its good emulsion-stabilizing properties. Absolute ethanol extract, on the other hand, presented a  $\zeta$ -potential of  $-37.5$  mV, despite its limited emulsifying ability.

Carboxylic acid groups with typical pKa values of  $\sim 3.5$  may be responsible for the negative  $\zeta$ -potentials of emulsions stabilized by OOC extracts [3]. This group would be fully charged ( $\text{COO}^-$ ) at pH 7, hence the strong negative charge of emulsion droplets. Proteins can be also responsible for the negative surface charge observed in our conditions as they usually show negative  $\zeta$ -potentials of approximately  $-40$  mV at pH 7 [1,34].

### 3.3. Effect of OOC 80% Extract Concentration and Oil Mass Fraction on Emulsion Formation

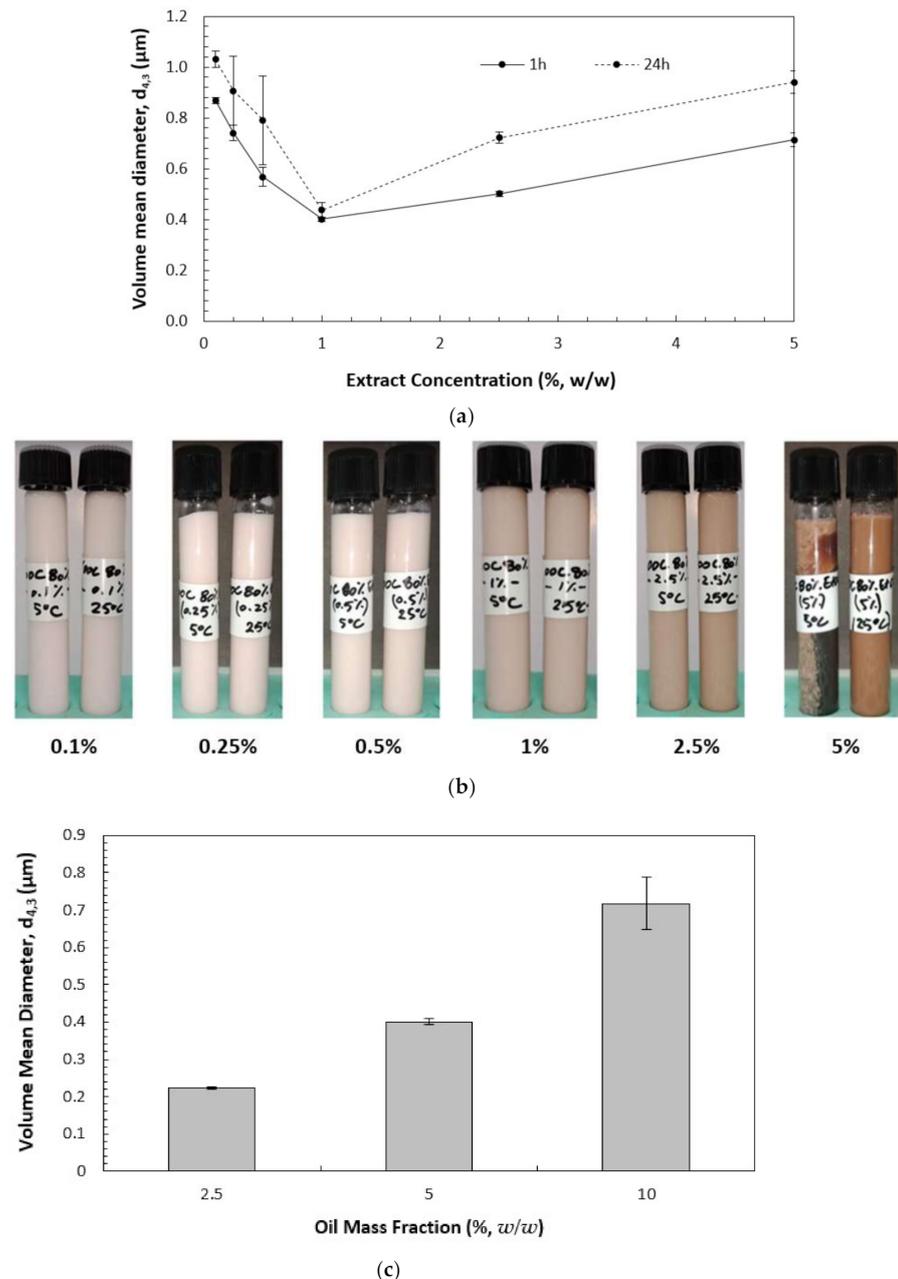
The  $d_{4,3}$  of emulsions gradually decreased upon increasing 80% (*w/w*) aqueous ethanolic extract concentration from 0.1 to 1% (*w/w*). Further increase in extract concentration appreciably increased the droplet size of emulsions and resulted in visible creaming at 5% (*w/w*) (Figure 5a,b). The ability of an emulsifier to produce small droplet size emulsions depends on (i) its ability to reduce the interfacial tension, (ii) the speed at which it adsorbs on the droplets' interface and (iii) its effectiveness to produce repulsive forces during homogenization [3,35]. Lower concentrations of OOC extract were insufficient, therefore, to reduce interfacial tension and/or stabilize the newly generated interfaces, while higher concentrations increased the contents of emulsion destabilizing agents (e.g., minerals) in the emulsions.

Increasing oil mass fraction from 2.5 to 10% (*w/w*) also increased the droplet size of OOC-stabilized emulsions (Figure 5c). This suggests that relatively high emulsifier-to-oil ratios are needed to produce small droplet size emulsions using the present extract. Moreover, further increase in oil concentration increases the viscosity of emulsions, which affects their disruption efficiency during homogenization [35]. Overall, small droplet size O/W emulsions can be prepared using 1% (*w/w*) OOC extract and 5% (*w/w*) soybean oil by high-pressure homogenization (100 MPa, four passes). We proceeded, therefore, to evaluate the stability characteristics of these emulsions at different stress conditions to determine their potential application.

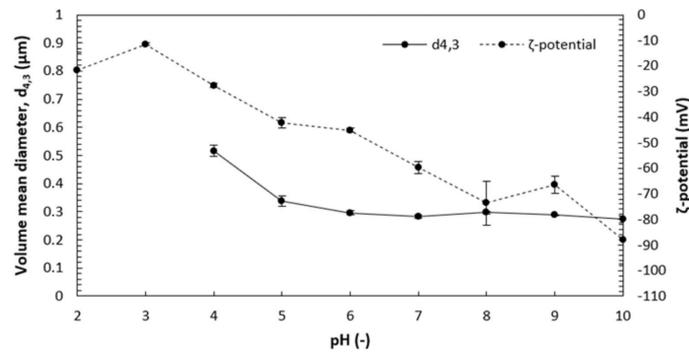
### 3.4. Effect of Stress Conditions on OOC 80% Extract Stabilized Emulsions

Emulsions prepared using OOC 80% (*w/w*) aqueous ethanol extract were stable from pH 5 to 10 with average  $d_{4,3}$  of 0.3  $\mu\text{m}$ . Further decrease in pH gradually increased the droplet size of emulsions or resulted in excessive creaming at pH 3 and 2 (Figure 6). Increasing NaCl concentration also reduced the stability of emulsions as indicated by droplet size measurements and visual appearance. The  $d_{4,3}$  increased to 6.2  $\mu\text{m}$  at 100 mM and visible creaming was observed from 25 mM (Figure 7). These results agreed with the  $\zeta$ -potential measurements of emulsions, which gradually decreased upon reducing the pH or increasing NaCl concentration (Figures 6 and 7).

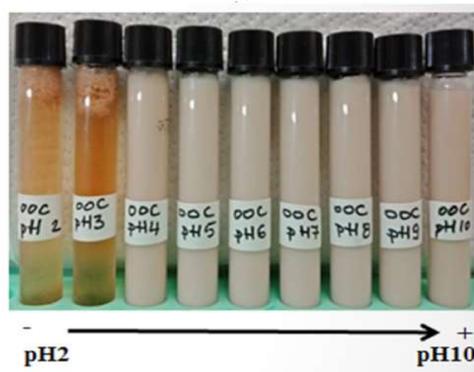
Saponins show a pKa of approximately 3.25 and, therefore, reducing the pH below this value leads to screening the surface charge of emulsions droplets [2]. Moreover, electrostatic screening of the negative charge of saponins can affect their intermolecular interaction with other components, including proteins, affecting the interfacial layer composition of emulsions droplets. The stabilization mechanism of OOC 80% extract-stabilized emulsions depends, therefore, importantly on electrostatic repulsion due to the adsorption of ionizable surface active components with ionizable groups, such as saponins. The contribution of other components (e.g., proteins) to emulsions stability is also suggested, explaining their resistance to complete phase separation (oiling-off) at extreme environmental conditions by providing stronger interfacial coverage.



**Figure 5.** (a) Effect of OOC 80% (*w/w*) aqueous ethanolic extract concentration on the volume mean droplet diameter of emulsions after 1 and 24 h of storage at 5 °C; (b) effect of OOC 80% extract concentration on the visual appearance of emulsions after 24 h of storage at 5 °C. (c) Effect of oil mass fraction on the mean droplet size of emulsions after 24 h of storage at 5 °C.

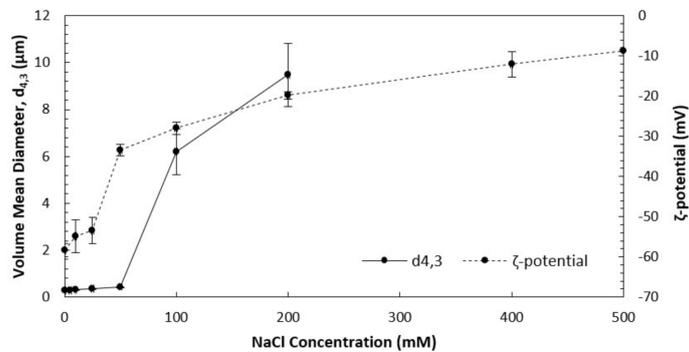


(a)

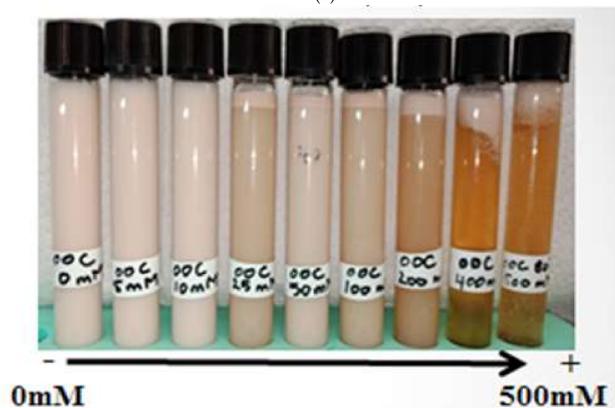


(b)

**Figure 6.** (a) Effect of pH on the volume mean droplet diameter ( $d_{4,3}$ ),  $\zeta$ -potential and (b) visual appearance of O/W emulsions prepared using OOC 80% aqueous ethanolic extract.



(a)



(b)

**Figure 7.** (a) Effect of NaCl concentration on the volume mean droplet diameter ( $d_{4,3}$ ),  $\zeta$ -potential and (b) visual appearance of O/W emulsions prepared using OOC 80% aqueous ethanolic extract.

The  $d_{4,3}$  of OOC 80% extract-stabilized emulsions did not change after 30 days of storage at different temperatures (Table 2), thus its potential use for the preparation of stable O/W emulsions by high-pressure homogenization.

**Table 2.** Volume mean droplet diameter ( $\mu\text{m}$ ) changes of emulsions prepared using 1% (*w/w*) OOC 80% extract and 5% (*w/w*) soybean oil, by high-pressure homogenization.

	5 °C	25 °C	50 °C
Day 1	0.437	0.405	0.401
Day 7	0.416	0.440	0.470
Day 15	0.427	0.405	0.481
Day 30	0.394	0.387	0.426

#### 4. Conclusions

The present work suggests a novel approach for using crude aqueous-ethanolic extracts from untreated olive oil cake to produce stable O/W emulsions with smaller droplet sizes and for an extended period of storage. OOC extracts successfully formed emulsions with average droplet sizes ( $d_{4,3}$ ) of 0.28–0.4  $\mu\text{m}$ . OOC 80% (*w/w*) aqueous ethanolic extract was selected, as it provided the best storage stability for up to 15 days at 25 °C. Our results clearly showed that this extract could produce emulsions with good physical stability at a wide range of pH (5–10) and when stored at 5, 25 and 50 °C for 30 days. However, the emulsions were highly unstable at extreme acidic pH (<4), and at increased ionic strengths ( $\geq 25$  mM). The main stabilization mechanism is believed to be mainly electrostatic, likely due to the presence of surface-active compounds with ionizable groups such as saponins. In future studies, it would be helpful to extend this work by evaluating other parameters, such as the taste profile, the toxicity, the cost, and the reliability of supply to encourage the application of this extract as a food emulsifier.

**Author Contributions:** Conceptualization, F.F., A.H. and M.N.; methodology, F.F.; software, F.F.; validation, A.H., M.A.N., and M.N.; formal analysis, F.F.; investigation, F.F. and F.B.; resources, M.N.; data curation, F.F.; writing—original draft preparation, F.F.; writing—review and editing, N.T.; visualization, F.F.; supervision, N.T.; project administration, M.N., A.H. and H.I.; funding acquisition, M.N. All authors have read and agreed to the published version of the manuscript.

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