PAPER

SCREENING OF EXTRA VIRGIN OLIVE OIL-IN-BITTER ORANGE JUICE (O/W) NANO-EMULSIONS STABILIZED WITH DIFFERENT FOOD-GRADE SURFACTANTS: A MODEL SYSTEM FOR NATURAL DAILY USE SALAD DRESSING

Ş. YALÇINÖZ*[1], E. ERÇELEBI[1], C. SOLANS[2,3] and T. TADROS[4]

[1]Food Engineering Department, Gaziantep University, Gaziantep-27310, Turkey

[2]Institut de Química Avançada de Catalunya (IQAC-CSIC). Jordi Girona 18-26, 08034 Barcelona, Spain

[3]Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Barcelona, Spain

[4]Independent researcher

*Corresponding author: selalekara@gantep.edu.tr

ABSTRACT

Olive oil-in-bitter orange juice (O/W) nano-emulsions were prepared by phase inversion composition method at 25°C. The emulsions were formulated with extra virgin olive oil as an organic phase, bitter orange juice (pH 2.57) as an aqueous phase and binary combinations of polyoxyethylene sorbitan monooleate, sorbitan monooleate, sucrose monopalmitate and sunflower lecithin as surfactants. Visual appearance, transparency, microstructure and particle size distribution of the nano-emulsions were influenced by surfactant blend composition and concentration. The current study may promote usage of bitter orange for flavoring and acidifying salads via increasing consumer awareness, and to promote being fit with daily diet routines.

Keywords: bitter orange, nano-emulsion, olive oil, salad dressing, surfactant

1. INTRODUCTION

Because of the long-term kinetic stability of nano-emulsions against physical effects such as temperature changes and dilution, their popularity and usage demands increase day by day (ANTON *et al.*, 2008). Their good resistance to gravity separation, coalescence and flocculation makes them incomparable and they are regarded as achieving ‘approaches thermodynamic stability’ (TADROS *et al.*, 2004). When the current food grade nano-emulsion studies in the literature are examined, most of them are studied under ideal conditions (that is, low density essential oils such as orange oil, lemon oil and etc. as an organic phase and buffer solutions with neutral pH as an aqueous phase) by using high energy methods and the characteristic properties of the emulsions such as particle size, physical properties and stability are examined (CHOI *et al.*, 2011; RAO and MCCLEMENTS, 2011; QIAN and MCCLEMENTS, 2011; RAO and MCCLEMENTS, 2012a; KALTSA *et al.*, 2013). So, nano-emulsion studies related to real food systems is lacking.
The phase inversion composition (PIC) method, which is one of the low energy emulsification methods, uses chemical energy liberated by the phase transition occurring while the emulsification procedure at constant temperature (SOLANS and SOLÉ, 2012). PIC procedure includes the introducing of an aqueous phase into an agitating organic phase. That is, the surfactant and oil constituents which making up the organic phase are uniformly blended together to get homogeneous solution. The aqueous phase is then introduced into the continuously stirring organic phase at a controlled injection rate (i.e., injection amount per time) (KOMAIKO and MCCLEMENTS, 2016). Considering the PIC method in detail to understand how the phase transitions are led by variation in composition during emulsification process itself at constant temperature, SOLANS and SOLÉ (2012) reported that when the aqueous phase is initially introduced into the organic phase, water-in-oil (w/o) emulsion is produced. As more aqueous phase is introduced, a liquid crystalline phase may be presented which can be so viscous to inhibit further stirring. As extra aqueous phase is introduced, a multiple emulsion (oil-in-water-in-oil, o/w/o) is fabricated, and the viscosity of the system diminishes. As the volume fraction of aqueous phase further increases, a transitional phase inversion happens (o/w/o to o/w), and the small oil droplets available within the aqueous phase of the o/w/o emulsions are liberated (KOMAIKO and MCCLEMENTS, 2016). Although studies have shown that low energy methods are generally more effective in producing smaller droplet sizes than high energy methods; the low energy methods are still not common in the food industry and also, the factors affecting the performance of low energy methods are still not fully understood (SABERI *et al.*, 2013).
Olive oil, as an organic phase of the current study, is high nutritious and beneficial to human health with its unique fatty acid composition, as well as high stability to lipid oxidation. However, finely dispersed olive oil droplets within emulsions have been examined usually over enzymatic reactions and, studies relating finely dispersed oil droplets within real food-grade emulsion systems are lacking (POLYCHNIATOU and TZIA, 2014). The bitter orange (*Citrus aurantium*), whose juice is the aqueous phase of the current study, is a fruit known with strong natural sour flavor due to the presence of beneficial flavonoids, mostly naringin, and neohespiridin, and due to its own tissue acidity (PETERSON *et al.*, 2006). Bitter orange has received great interest recently for its use in the prevention of major health challenge of 21^{st} century: obesity. *p*-sinephrine, found in unripe bitter orange (*Citrus aurantium*) fruit or shell extract, is commonly used to control weight, weight loss and strengthen stamina in sports performance products (STOHS *et al.*, 2012; STOHS *et al.*, 2011). American Food and Drug Administration on 11 April 2004

restricted the use of anti-obesity products containing ephedrine since the possible health problems related to them such as vascular occlusion, hypertension, stroke, psychiatric problems. Thus, bitter orange has become a safe alternative to ephedrine, and the importance of the use of bitter orange in dietary products has increased (TOKGOZ and GÖLCÜKLÜ, 2009). Bitter orange juice can be alternative to lemon juice for flavoring and acidifying salads (KARABIYIKLI *et al.*, 2014), which may promote being fit with easy daily diet routines. Since bitter orange is produced large quantities in the Mediterranean region, the use of bitter orange could be expanded by increasing consumer awareness. Hovewer, there has been no detailed research and data on the use of bitter orange juice in food systems, especially no studies have been done on olive oil-in-bitter orange juice emulsions at nano-scale. Therefore, current study has emerged from the intention of expanding usage of bitter orange for flavoring and acidifying salads via increasing consumer awareness, and to promote being fit with daily diet routines. Olive oil-in-bitter orange juice (O/W) nano-emulsions were prepared by phase inversion composition (PIC) method with binary combinations different surfactants. Visual appearance, transparency, emulsion stability index, microstructure and particle size distribution of emulsions were investigated.

2. MATERIALS AND METHODS

2.1. Materials

Bitter oranges (*Citrus aurantium* L.) were harvested at optimum maturity in February 2017, from Mersin, Çukurova region, in Turkey. Polyoxyethylene sorbitan monooleate (Tween 80) (Hydrophilic–lipophilic balance (HLB) =15.0), Sorbitan monooleate (Span 20) (HLB=8.6), Sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Chemical Company. Sucrose Monopalmitate (SMP) (HLB=18.5) and Sunflower Lecithin (SL) were kindly gifted from Compass Foods Company (Singapore). Since HLB of SL was not reported in literature, also in product specifications, it was assumed to have similar HLB of sunflower and soybean lecithin,i.e., HLB=8.0 (CHEN *et al.*, 2015). Commercially available Turkish extra virgin olive oil (EVOO) Tariş was obtained from the local supermarkets of Gaziantep/Turkey and used without further purification. Ultrapure water from a Milli-Q Plus system was used for dilution of bitter orange juice concentrates.

2.2. Preparation of nano-emulsions by low energy method

Oil-in-water (O/W) nano-emulsions with 90 % aqueous phase and 10 % organic phase (surfactant blend + olive oil) were prepared by a low energy method of Phase Inversion Composition (PIC). Four food-grade surfactants, namely, two types of low molecular weight surfactants (Polyoxyethylene sorbitan monooleate, Tween80, and Sorbitan monooleate, Span20), one sucrose ester (Sucrose monopalmitate, SMP) and one lipid based surfactant (Sunflower lecithin, SL) were selected to stabilize the nano-emulsions as binary combinations of Tween80/Span20, Tween80/SL, Tween80/SMP and SMP/SL. Extra virgin olive oil (EVOO) was used as an organic phase. Bitter orange juice was centrifugated at 5000 rpm for 60 min at three times, filtered using 20 micron filter paper, which was used as aqueous phase.

Prescreening tests: To define adequate surfactant blend (S_A/S_B) weight ratios (w/w) and oil to surfactant blend (O/S, where S stand for S_A+S_B) weight ratios (w/w), nano-emulsions were prepared at many O/S weight ratios with changing surfactant blend (S_A/S_B) weight ratios (w/w). Due to the PIC method, first surfactant blends of Tween80/Span20, Tween80/SL, Tween80/SMP and SMP/SL were prepared by blending with weight ratios of (S_A/S_B) 90/10, 85/15, 80/20, 75/25 at ambient temperature. A certain amount of EVOO, was evenly mixed with surfactant blends at O/S weight ratios of 10/90, 15/85, 20/80, and 25/75 under a stirring rate of 500 rpm via vortex (Velp Scientifica, Europe). The final mass of organic phase was 0.4 grams. Finally, fixed amount of bitter orange juice as an aqueous phase (3.6 grams) was continuously added to the organic phase by a disposable plastic syringe (5mL) at the injection rate of 1mL per minute and, stirring rate of 2400 rpm was maintained in the meantime. These nano-emulsions observed after 24 hour storage at 25 °C to evaluate visual physical stability (no creaming or phase separation after one night stand). In the light of prescreening tests, to test the effect of surfactant blend composition and O/S weight ratio on transparency, microstructure and particle size distribution of nano-emulsions, nano-emulsions were prepared at many O/S weight ratios of 10/90, 15/85, 20/80, and 25/75 with constant surfactant blend (S_A/S_B) weight ratio of 90/10.

2.3. Visual appearance

Visual appearances of nano-emulsions after 24 hour storage at 25°C were captured by Nikon 5300 camera.

2.4. Transparency analysis

Transparency of extra virgin olive oil-in-bitter orange juice was determined by recording the absorbance at 600 nm, using a UV-VIS spectrophotometer (SP-3000nano, OPTIMA, Tokyo, Japan). The transparency was calculated by the following equation:

$$T = 1/10^{A} \tag{1}$$

where T is the transparency and A is the value of absorbance at 600 nm. A high T value would represent a transparent appearance (HA *et al.*, 2015). Transparency results are given as a percentage.

2.5. Emulsion stability index

Emulsion stability index (ESI) of the emulsions was determined by turbidimetrically (WANG *et al.*, 2008). An aliquot (50 μL) of freshly prepared emulsion was taken from the bottom of emulsion, immediately (0 min) and 10 min after preparation and, diluted (1:10, v/v) in 0.1% (w/v) SDS solution. The absorbance of the diluted emulsion at 0 and 10 min was measured at 500 nm by UV-VIS spectrophotometer (SP-3000nano, OPTIMA, Tokyo, Japan). ESI values were calculated by the following equation:

$$\text{ESI (min)} = \frac{\text{Ao}}{\text{Ao} - \text{A}_{10}} \times 10 \tag{2}$$

where A_0 and A_{10} are the absorbance of the diluted emulsion at 0 and 10 min, respectively. These measurements were performed in triplicate.

2.6. Raman microscopy

Droplet images for the emulsions were captured under Raman microscope at room temperature. 50 μm of freshly made nano-emulsion was placed on a 1.2 mm thick glass slide without a coverslip, photomicrographs (20X magnification) were taken using A Renishaw InVia Raman Microscope (Renishaw Plc., U.K.) equipped with a Leica ×20 objective lens.

2.7. Particle size distribution

The particle size distribution (PSD) of the emulsions was measured using a laser light scattering instrument (Partica LA-950, Horiba Ltd., Japan). The device could detect particle sizes ranging from 10 nm to 3 mm. To avoid multiple scattering effects, emulsions were diluted with distilled water. Particle size measurements were reported as Sauter, or surface mean diameters D_{32} ($D_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$) or volume mean diameters D_{43} ($D_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$), where n_i is the number of particles with diameter d_i. The refractive indices of the dispersed and continuous phases used in the calculations of the PSD were 1.135 and 1.330, respectively. To determine the width of the distribution of particle sizes, 'span' was calculated from the following formula:

$$\text{Span} = [d\,(v, 90) - d\,(v, 10)] \;/\; d\,(v, 50) \qquad (3)$$

In this formula, d (v, 10), d (v, 50), and d (v, 90) are diameters at 10, 50, and 90% cumulative volume, respectively. In other words, [d (v, 90) – d (v, 10)] is the range of the data and d (v,50) is the median diameter (MAHDI JAFARI *et al.*, 2006).

2.8. Statistical analyses

All measurements were performed on freshly prepared samples, and were reported as means and standard deviations (n=2). This study is designated as a 4 (number of surfactant blend compositions) x 4 (number of oil to surfactant blend weight ratios) factorial structure (4x4). Experimental data were subjected to two-way analysis of variance and the means were compared by Tukey multiple range test at $p < 0.05$ significance level using SPSS version 22 (SPSS Inc., Chicago, IL, USA). Pearson's correlation test was used to determine point- biserial correlation among experimental parameters. Sigma Plot (Sigma Plot 10.0 Windows version, SPSS Inc.) is used for graph preparation.

3. RESULTS AND DISCUSSION

Initial evaluation of freshly prepared nano-emulsions was made by prescreening tests to define adequate surfactant blend (S_A/S_B) weight ratios and oil to surfactant blend weight ratios for obtaining clear, translucent nano-emulsions granted with good physical stability. In prescreening tests, nano-emulsions were prepared at many O/S weight ratios with changing surfactant blend (S_A/S_B) weight ratios were observed after 24 hour storage at 25 °C to evaluate visual physical stability against gravitational seperation. Table 1 shows results of the prescreening tests. Table 1 clearly indicates that all nano-emulsion formulations prepared with O/S weight ratios of 10/90, 15/85 along with 90/10 surfactant mixing ratio (S_A/S_B= 90/10) resulted in clear, translucent nano-emulsions with good physical stability without creaming or phase separation after one night stand at 25°C . In the light of prescreening tests and to make system less complex, to test the effect of surfactant blend composition and O/S weight ratio on transparency, microstructure and particle size distribution of nano-emulsions, nano-emulsions were prepared at many O/S weight ratios of 10/90, 15/85, 20/80, and 25/75 with constant surfactant blend (S_A/S_B) weight ratio of 90/10.

For many industrial applications optical transparency of emulsion-based delivery system is quite important (QIAN and MCCLEMENTS, 2011; ULUATA *et al.*, 2016), so relationship between O/S weight ratio and transparency of nano-emulsions with respect to surfactant blend composition were represented in Fig. 1.

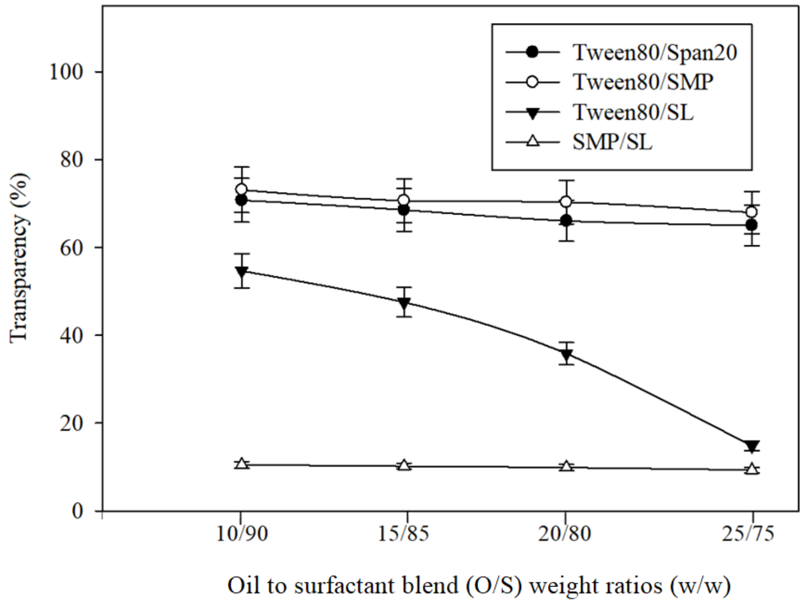

Figure 1. Change in the transparency of extra virgin olive oil-in-bitter orange juice nano-emulsions prepared with different surfactant blend compositions (Constant S_A/S_B= 90/10) as a function of O/S weight ratio (O/S= 10/90, 15/85, 20/80 and 25/75). (Bars indicate the standard deviations).

Statistical analyses revealed that transparency of nano-emulsions was significantly (p<0.05) affected by both main factors (Surfactant blend composition and O/S weight ratio) and two way interaction of these factors (Surfactant blend composition and O/S weight ratio) (Table 2). For all surfactant combinations, nano-emulsions prepared at 10/90 O/S weight ratio exhibited the highest transparency. As the surfactant concentration decreased with increasing O/S weight ratio from 10/90 to 25/75, emulsions became less translucent even milky. These findings have good agreement with photographs of nano-emulsions (Table 1). Nano-emulsions prepared with binary combinations of Tween80/Span20 and Tween80/SMP showed the highest transparency, over than 65 % (Fig. 1) and, similar decreasing trend was observed as the surfactant concentration decreased. Most pronounced change in transparency was seen in emulsions stabilized with Tween80/SL. The change can be clearly observed as transformation from transparent appearance to the milky one with the increase in the O/S weight ratio (Table 1).

Transparency of emulsions prepared with SMP/SL showed the lowest transparency with almost no change as O/S weight ratio change. Pearson's test showed significant negative correlation between surfactant blend composition and transparency, (r= -0.885; p<0.01) (Table 3). Statistical analyses also revealed that D_{32} and D_{43} of nano-emulsions were significantly (p<0.05) influenced by both main factors (Surfactant blend composition and O/S weight ratio) and two way interaction of these factors (Surfactant blend composition and O/S weight ratio) (Table 2). There was positive correlation between surfactant blend composition and D_{32} (r= 0.807; p<0.01) and D_{43} (r= 0.731; p<0.01) of emulsions, respectively, due to the Pearson's test (Table 3). Surfactant concentration has a direct effect on the particle size, which in turn affects turbidity of the emulsion. As the surfactant concentration increase, generally particle size of the emulsion droplets decreases (Table 4). Small emulsion droplets are skilled to scatter light less efficiently compared to large ones (QIAN and MCCLEMENTS, 2011) and, when looking under a white light source, nano-emulsions with small droplets appear transparent with a reddish tinge (MASON *et al.*, 2006), which accounts for the higher transparency of the nano-emulsion with small droplet sizes. At higher droplet sizes, nano-emulsions appear milky due to the strong multiple scattering of light (MASON *et al.*, 2006). So, nano-emulsion can be seen transparent or semi-transparent due to their small particle diameters (SOLANS *et al.*, 2005; PEY *et al.*, 2006) and even milky up to 500 nm (PORRAS *et al.*, 2008), which supports our findings in Table 1. In literature, several authors reported increased turbidity as the particle size of the emulsion increases (QIAN and MCCLEMENTS, 2011; SABERI *et al.*, 2013; ULUATA *et al.*, 2016).

Effects of O/S weight ratio on emulsion stability index (ESI) were expressed as the change in absorbance during 10 min interval and shown in Fig. 2. As shown from Fig. 2, generally, ESI of nano-emulsions stabilized with Tween80/Span20, Tween80/SL, and SMP/SL decreased as the surfactant concentration increased with decreasing O/S weight ratio. However, for the emulsions prepared with Tween80/SMP did not show a definite trend with change in O/S weight ratio. This is probably caused by change in emulsion turbidity, either raise or reduces, with rising particle size compared to initial particle size (MCCLEMENTS, 2007). Moreover, MCCLEMENTS (2007) states that there is no simple mathematical relation between particle size and turbidity, particularly in region where the particle radius is almost equivalent to the wavelength of light, which may accounted for fluctuations in ESI. Moreover, statistical analyses also revealed that only O/S weight ratio had statistically significant (p < 0.05) effect on ESI of nano-emulsions (Table 2). Pearson's test showed significant negative correlation between O/S weight ratio and ESI, (r= -0.523; p < 0.01) (Table 3).

Microscopic photographs of emulsions were taken using Raman microscope with 20X magnification. Fig. 3 represented the microstructures of extra virgin olive oil-in-bitter orange juice nano-emulsions formulated with different compositions. Fig. 3 indicated that surfactant blend composition and O/S weight ratio did have appreciable effect on microstructure. Emulsions prepared with Tween80/Span20, Tween80/SMP, and Tween80/SL showed similar spherical droplet structure. The particle size of these nano-emulsions gets smaller and more distributed as the surfactant concentration increases with decreasing O/S weight ratio, shift from O/S of 25/75 to 10/90. Emulsions prepared with SMP/SL showed completely different structure compared to emulsions prepared blends of Tween80/Span20, Tween80/SMP, and Tween80/SL.

Table 1. Photographs of bitter orange juice/surfactant blend (S_A+S_B)/ olive oil (EVOO) % organic phase (surfactant blend + olive oil). O/S = 10/90, 15/85, 20/80 and 25/75. S_A/S_B = 90/10, 85/15, 80/20 and 75/25.

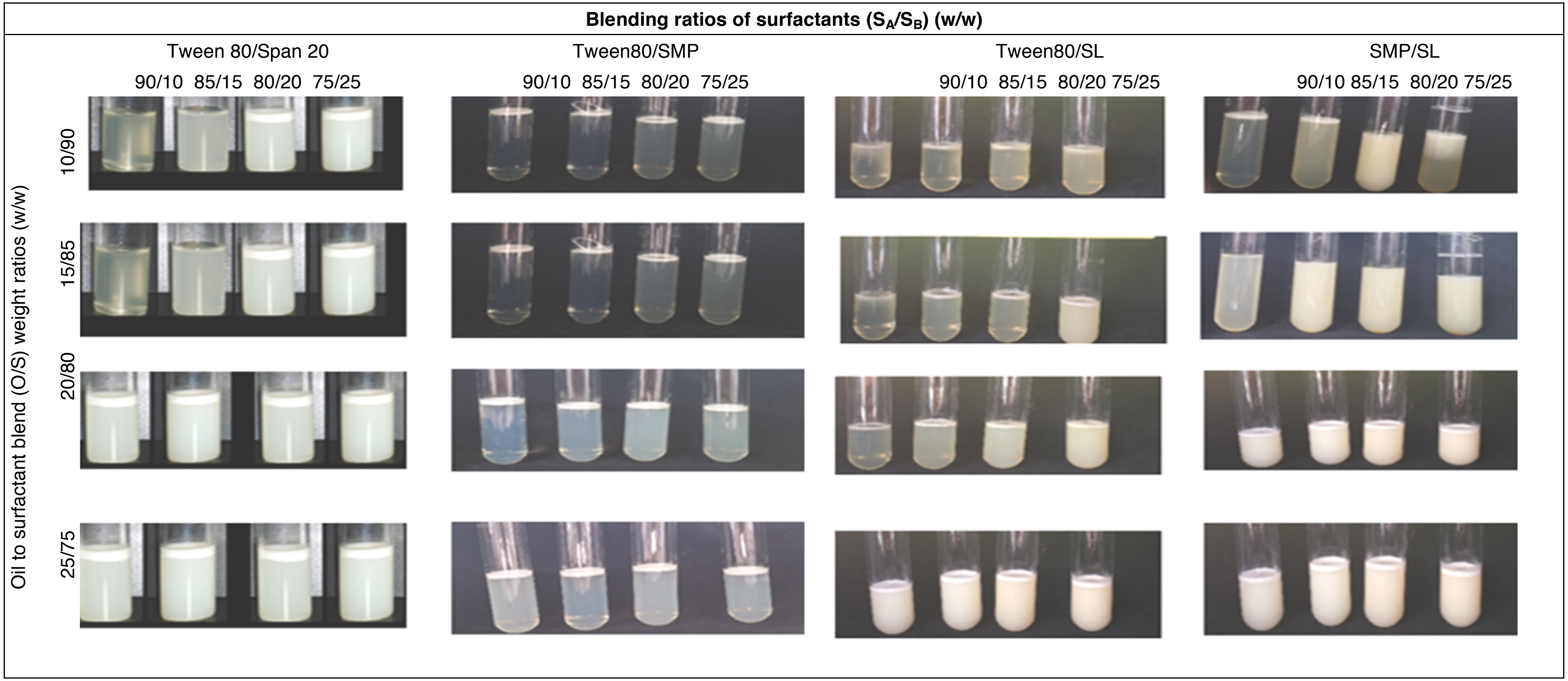

Table 2. Mean squares from two-way analysis of variance results of extra virgin olive oil-in-bitter orange juice nano-emulsions prepared with different surfactant blend compositions (Constant S_A/S_B= 90/10) and O/S weight ratios (O/S=10/90, 15/85, 20/80 and 25/75).

Source of variation	DF	Transparency (%)	ESI (min)	Area-based droplet size (D_{32})	Volume-based droplet size (D_{43})	Span (Dimensionless)
Main effects						
Surfactant blend composition	3	6464.561*	173.272	3655430.031*	8988824.365*	83.534
O/S weight ratio (w/w)	3	249.759*	281.385*	568763.948*	1590930.281*	85.789
Two-way interaction						
Surfactant blend composition*O/S weight ratio	9	126.457*	72.934	239800.642*	1345705.087*	29.211
Error		14.161	64.791	4648.781	14157.406	64.739

*Significant at (p<0.05).

Table 3. Pearson's correlation results of extra virgin olive oil-in-bitter orange juice nano-emulsions prepared with different surfactant blend compositions (Constant S_A/S_B= 90/10) and O/S weight ratios (O/S=10/90, 15/85, 20/80 and 25/75).

	Surfactant blend composition	O/S weight ratio (w/w)	Transparency (%)	ESI (min)	Area-based droplet size (D_{32})	Volume-based droplet size (D_{43})	Span (Dimensionless)
Surfactant blend composition	1						
O/S weight ratio (w/w)	0.000	1					
Transparency (%)	-0.885**	-0.185	1				
ESI (min)	-0.094	-0.523**	0.419*	1			
Area-based droplet size (D_{32})	0.807**	0.328	-0.926**	-0.426*	1		
Volume-based droplet size (D_{43})	0.731**	0.322	-0.875**	-0.404*	0.945**	1	
Span (Dimensionless)	-0.142	-0.346	0.171	0.336	-0.262	-0.190	1

**Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed).

Clustered, nonhomogeneous and, irregular shaped droplets were seen in emulsions prepared with SMP/SL, as the surfactant concentration decreases from O/S weight ratios of 15/85 to 25/75, flocculation of emulsion particles increases, which may resulted from the lack of minimum surfactant concentration to hold the emulsion particles apart from each other.

If one compares all emulsion in terms of effect of O/S weight ratio over particle size without considering their surfactant blend compositions, as expected, an increase in O/S weight ratio, i.e., decreasing surfactant concentration from 10/90 to 25/75 resulted an increase in the particle size, D_{32} and D_{43} (Table 4). That is, smallest droplets were seen at 10/90 O/S weight ratio, while the highest droplets were seen at 25/75 O/S weight ratio. This probably caused by as the particle size decreases surface area increases, so it increases the extent of surfactant to hydrate the droplets. That is, increased adsorption of surfactant to oil-water interface thus more surfactant available to stabilize newly formed droplets which in turn facilitates formation of smaller droplets (CHOI *et al.*, 2011; SABERI *et al.*, 2013).

Table 4. Effect of O/S weight ratio on the emulsion particle size of extra virgin olive oil-in-bitter orange juice nano-emulsions prepared with different surfactant blend compositions (Constant S_A/S_B= 90/10). (O/S=10/90, 15/85, 20/80 and 25/75).

O/S	D_{32} (nm±SD)	D_{43}(nm±SD)	Span (dimensionless)
Tween80/Span20			
10/90	130±9.19a,A	1203±85.07a,A	23.84±1.69a,A
15/85	126±8.92a,B	143±10.13a,A	0.93±0.07 a,A
20/80	134±9.48a,C	155±10.93a,B	0.96±0.07 a,A
25/75	140±9.89a,D	157±11.09a,C	0.86±0.06 a,A
Tween80/SMP			
10/90	255±18.03b,A	311±22.01a,A	1.03±0.07 a,A
15/85	260±18.36b,B	348±24.60a,A	0.92±0.07 a,A
20/80	262±18.55b,C	356±25.19a,B	1.30±0.09 a,A
25/75	314±22.17b,D	373±26.38a,C	1.06±0.07 a,A
Tween80/SL			
10/90	163±11.54c,A	231±16.33b,A	1.40±0.10 a,A
15/85	242±17.11c,B	569±40.23b,A	1.34±0.09 a,A
20/80	655±47.02c,C	2018±142.70b,B	23.97±1.66 a,A
25/75	1730±122.33c,D	3079±217.73b,C	1.58±0.11 a,A
SMP/SL			
10/90	1171±82.77d,A	1350±95.47c,A	0.97±0.07 a,A
15/85	1642±116.10d,B	2574±181.97c,A	1.48±0.10 a,A
20/80	1690±119.52d,C	3146±222.47c,B	1.69±0.12 a,A
25/75	2022±142.99d,D	3317±234.56c,C	1.79±0.13 a,A

Mean values ± Standard deviation (n=2). Values in the same column followed by different lowercase letters (surfactant blend composition effect) indicate statistical difference at p<0.05 significance level among emulsions with change in surfactant blend composition at constant O/S weight ratio due to the Tukey multiple range test. Values in the same column followed by different uppercase letters (O/S weight ratio effect) indicate statistical difference at p<0.05 significance level among emulsions with change in O/S weight ratios at constant surfactant blend composition due to the Tukey multiple range test. Each column is evaluated within itself.

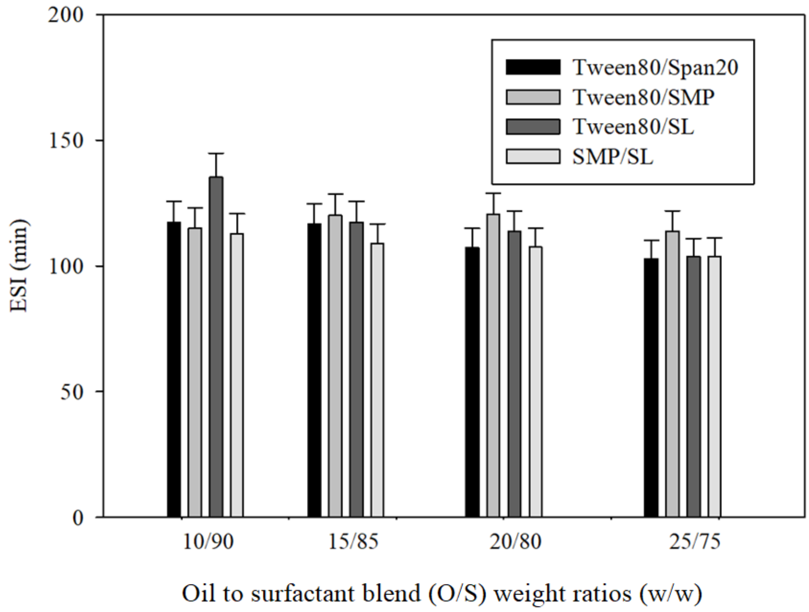

Figure 2. Change in ESI of extra virgin olive oil-in-bitter orange juice nano-emulsions formulated with different surfactant blend compositions (Constant S_A/S_B= 90/10 as a function of O/S weight ratio (O/S=10/90, 15/85, 20/80 and 25/75). (Bars indicate the standard deviations).

In literature several authors observed decrease in particle size of droplets with increase in surfactant concentration up to the certain levels (YUAN *et al.*, 2008; CHOI *et al.*, 2011; SABERI *et al.*, 2013; ULUATA *et al.*, 2016). The results presented Figs. 4 to 7 and given in Table 4, indicate that emulsion distribution getting narrower and span getting smaller as the surfactant concentration increased by reduction O/S weight ratio, which supports findings of (LOVELYN and ATTAMA, 2011), that is, the smaller the Span value, the narrower the particle size distribution. Statistical analyses showed that neither O/S weight ratio nor surfactant blend composition had statistically significant (p>0.05) effect on Span of nano-emulsions (Table 2).

These results can be explained by the different hydrophilicity of the surfactants. The hydrophilicity of surfactants is usually measured by their hydrophilic-lipophilic balance (HLB). Lipophilic surfactant has HLB number below 9.0, hydrophilic surfactant has HLB number above 11.0 and, theoretically, low HLB (3–6) surfactants are utilized in the preperation of W/O nano-emulsions while high HLB (8-18) surfactants are utilized in the preperation of O/W nano-emulsions (DAVIES, 1957). The HLB of a surfactant blend can be calculated by the summation of the product of HLB and weight percentage of each surfactant (CHEN *et al.*, 2015). HLB values of Tween80, Span 20, SMP and SL were 15.0, 8.6, 18.5 and 8.0, respectively. HLB values of the binary combinations of Tween80/Span20, Tween80/SMP, Tween 80/SL and SMP/SL used in the current study were calculated as 14.36, 15.35, 14.30, and 17.45 respectively. Contrary to theoretically expected, in theory it is supposed to have the lowest particle sizes in the o/w nano-emulsions composed of high hydrophilic surfactants (high HLB value) due to increased packing and stabilizing effect of the surfactant blend (TAN and NAKAJIMA, 2005), SMP/SL (HLB=17.45) nano-emulsions had the highest particle sizes. Also, even though the Tween80/Span20 (HLB=14.36) and Tween 80/SL (HLB=14.30) nano-emulsions had nearly same HLB value, Tween80/Span20 (HLB=14.36) emulsions result with the highest transparency (Table 1, Fig. 1) and relatively low particle diameters (Table 4) at O/S weight ratios of 15/85 and 10/90, while Tween 80/SL nano-emulsions was semi-transparent (Table 1) and were only in nano-scale at O/S weight ratios of 15/85 and 10/90 (Table 4). As stated by YALÇINÖZ and ERÇELEBİ (2018), although the HLB dictionary provides theoretically relevant information about the required system, it is essential to make experimental screening to test the actual suitability of the surfactants in the system.

These results can also be caused by the different physicochemical properties of the surfactants. Tweens are nonionic surfactants with a polyoxyethylene head group and a single hydrocarbon tail (MAHDI *et al.*, 2011). The hydrophobic tail of Tween 80 is curled resulting in maximum curvature and packing parameter to facilitate the preperation of nano-emulsions (KOMAIKO and MCCLEMENTS, 2016). Tween 80 is the most soluble surfactant among Tween series (MAHDI *et al.*, 2011). In the current study, all nano-emulsion contained equal amounts of Tween 80, so it was assumed that Tween 80 has the same effect to all nano-emulsions regardless of differences in co-surfactant composition. These results suggest that Tween80 was good worked with Span20, followed by SMP. MCCLEMENTS and RAO (2011) reported that Tweens and Spans are very conventional in food use due to their low toxicity, not being irritant, and facility to fit both high-energy and low-energy emulsification methods. Spans and Tweens work well for producing stable emulsions, even though the individual surfactants alone do not produce stable multiple emulsion systems (LU and RHODES, 2000), which supports our findings. In literature there are several studies using Spans and Tweens solely or as binary combinations (YUAN *et al.*, 2008; LEONG *et al.*, 2009; SILVA *et al.*, 2012; KUMAR DEY *et al.*, 2012; ABBAS *et al.*, 2013; PESHKOVSKY *et al.*, 2013).

Sucrose monopalmitate (SMP) is a hydrophilic, water soluble, nonionic surfactant, which has a polar head group (sucrose) and a nonpolar tail group (palmitate) (CHOI *et al.*, 2011). Recent studies revealed that SMP is not an effective surfactant to stabilize O/W nano-emulsions and emulsions under acidic pH when used solely (RAO and MCCLEMENTS, 2011; RAO and MCCLEMENTS, 2012b). In the current study, Tween80/SMP nano-emulsions showed good transparency (Table 1, Fig. 1) and fine droplets (Table 4) even under acidic pH (at pH 2.57). These results were also in good accordance with the studies of RAO and MCCLEMENTS (2012b) stating that acid stability of SMP stabilized nano-emulsions can be enhanced by blending SMP and Tween 80. Including, nowadays, surfactants of sugar esters are very popular in food due to their good taste and aroma profile, low toxicity, and high biodegradability compared to petrochemical-based surfactants (RAO and MCCLEMENTS, 2011).

Lecithin is a lipid mixture with phospholipids, which contains two nonpolar hydrocarbon chains and a zwitterionic polar head group with positive and negative charges deriving from amine and phosphate groups, respectively (CHEN *et al.*, 2015). Lecithin is very common emulsifying agent in food due to its low toxicity, biocompatibility, and generally recognized-as-safe regulatory status (CHEN *et al.*, 2015). Sunflower lecithin could take place of soybean lecithin due to being a non-GMO product (CABEZAS *et al.*, 2016). However, in the current study, particle sizes of nano-emulsions stabilized with Tween80/SL were only in nano-scale at 10/90 and 15/85 O/S weight ratio (Table 4).

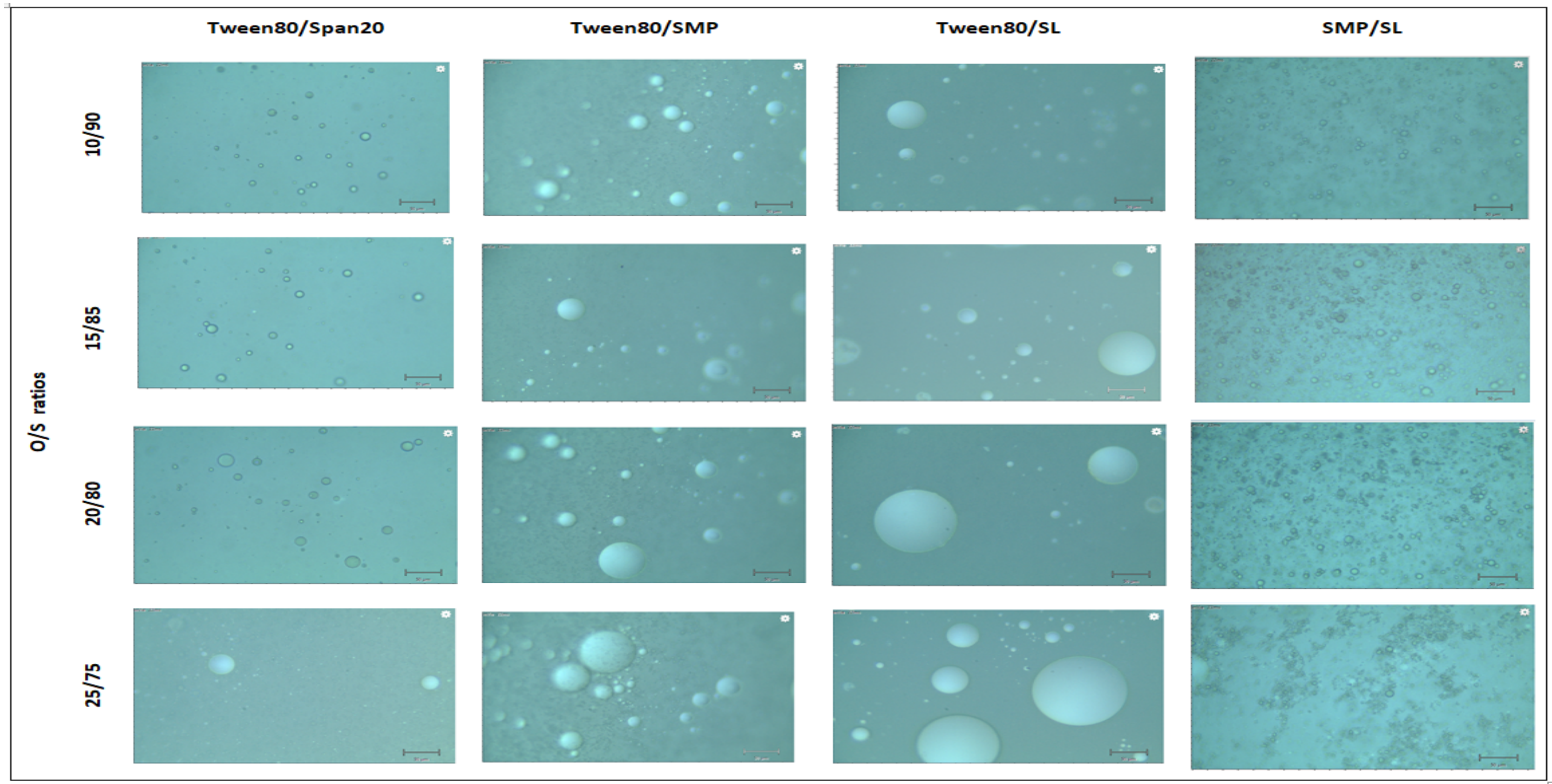

Figure 3. Micrographs of freshly prepared bitter orange juice/Surfactant blend (S_A+S_B)/ olive oil (EVOO) nano-emulsions at 25 °C, which were nano-emulsions with 90 % aqueous phase (at pH 2.57), i.e., 90% aqueous phase and 10 % organic phase (surfactant blend + olive oil). O/S weight ratios = 10/90, 15/85, 20/80 and 25/75. S_A/S_B = 90/10. The scale bars indicate 50 μm.

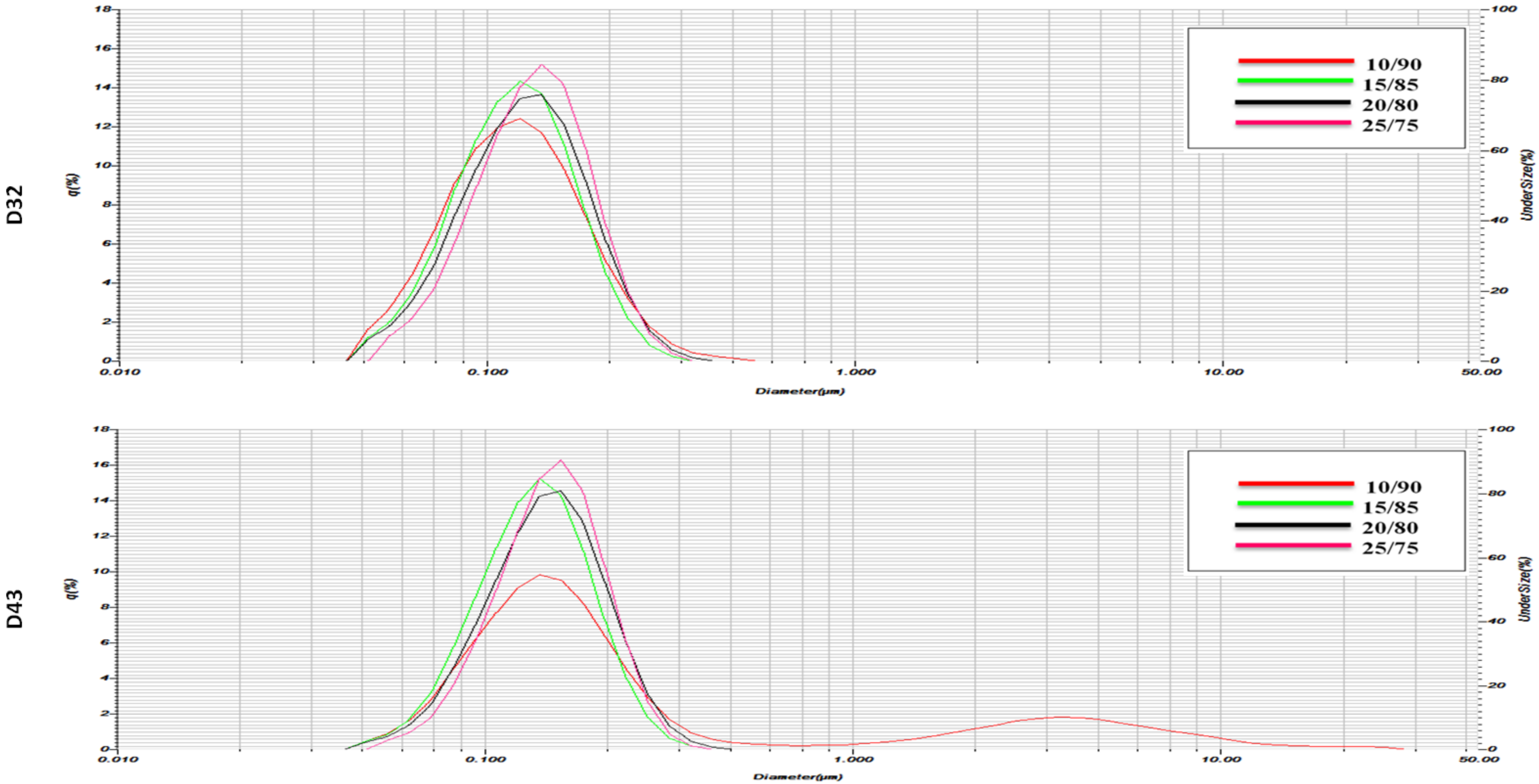

Figure 4. Particle size distribution graph of olive oil-in-bitter orange juice nano-emulsions prepared with Tween80/Span20 with constant surfactant mixing ratio (Tween80/Span20 = 90/10) as a function of O/S weight ratios of 10/90, 15/85, 20/80 and 25/75.

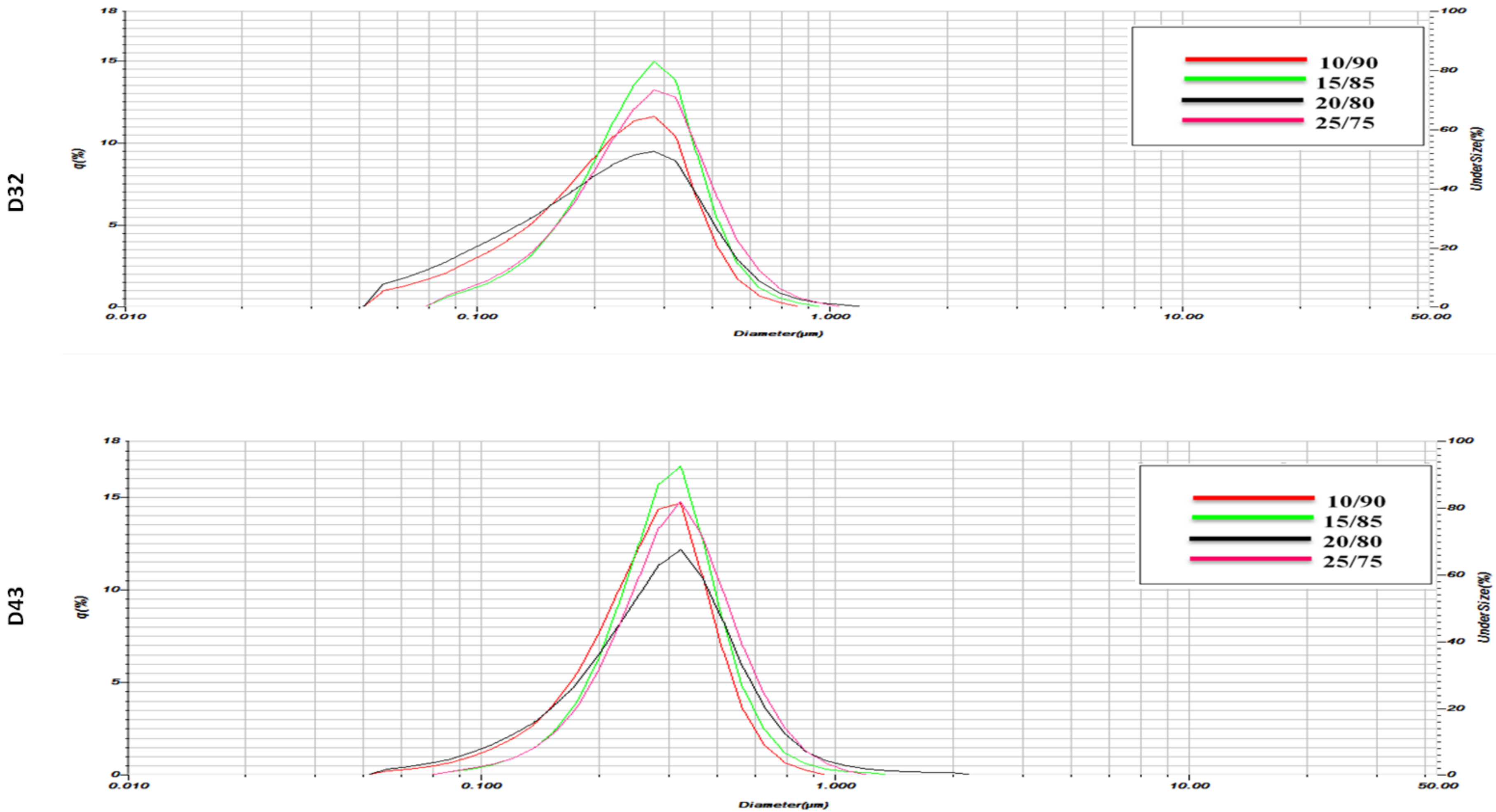

Figure 5. Particle size distribution of olive oil-in-bitter orange juice nano-emulsions prepared with Tween80/SMP with constant surfactant mixing ratio (Tween80/SMP = 90/10) as a function of O/S weight ratios of 10/90, 15/85, 20/80 and 25/75.

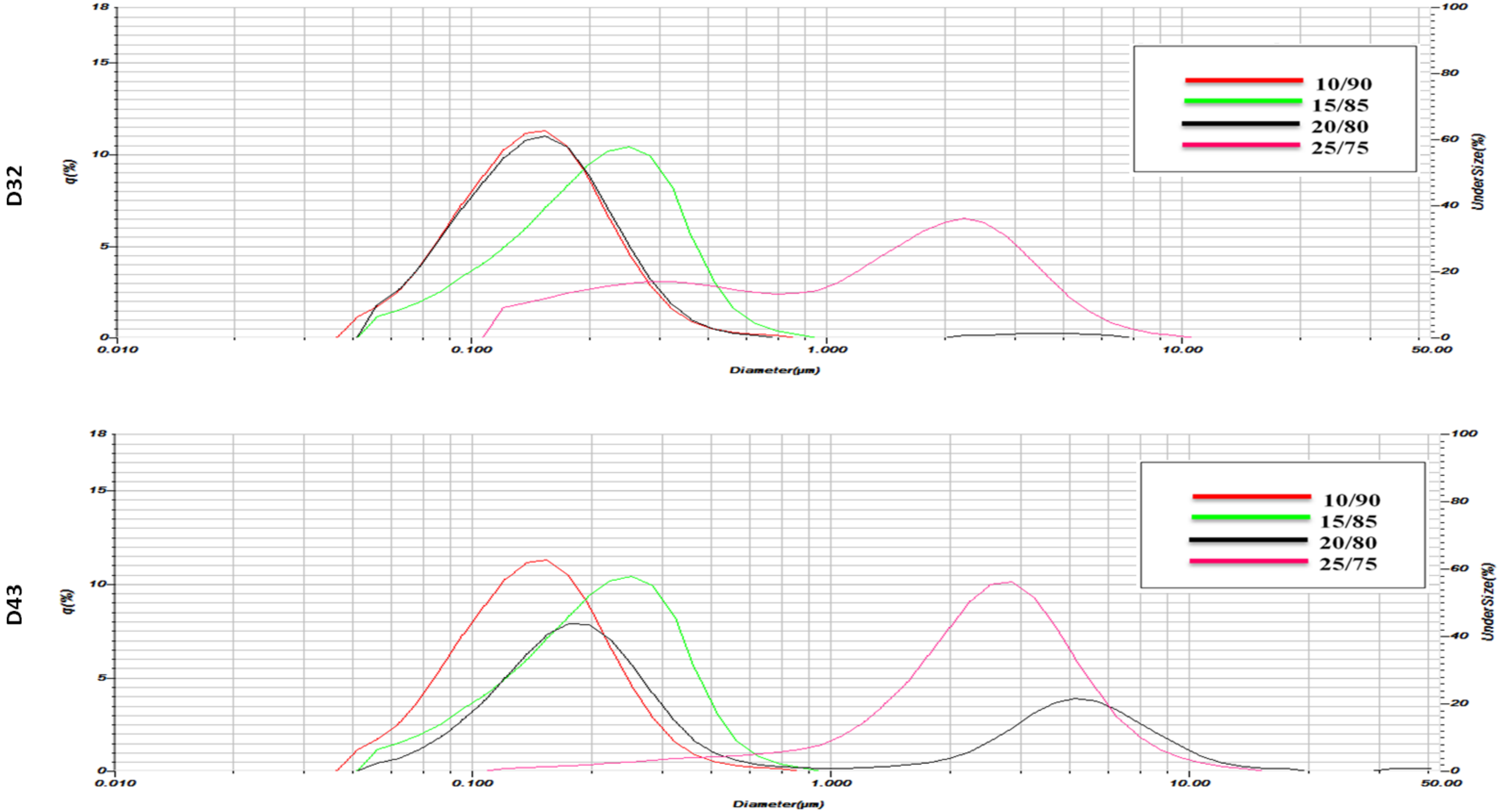

Figure 6. Particle size distribution graph of olive oil-in-bitter orange juice nano-emulsions prepared with Tween80/SL with constant surfactant mixing ratio (Tween80/SL = 90/10) as a function of O/S weight ratios of 10/90, 15/85, 20/80 and 25/75.

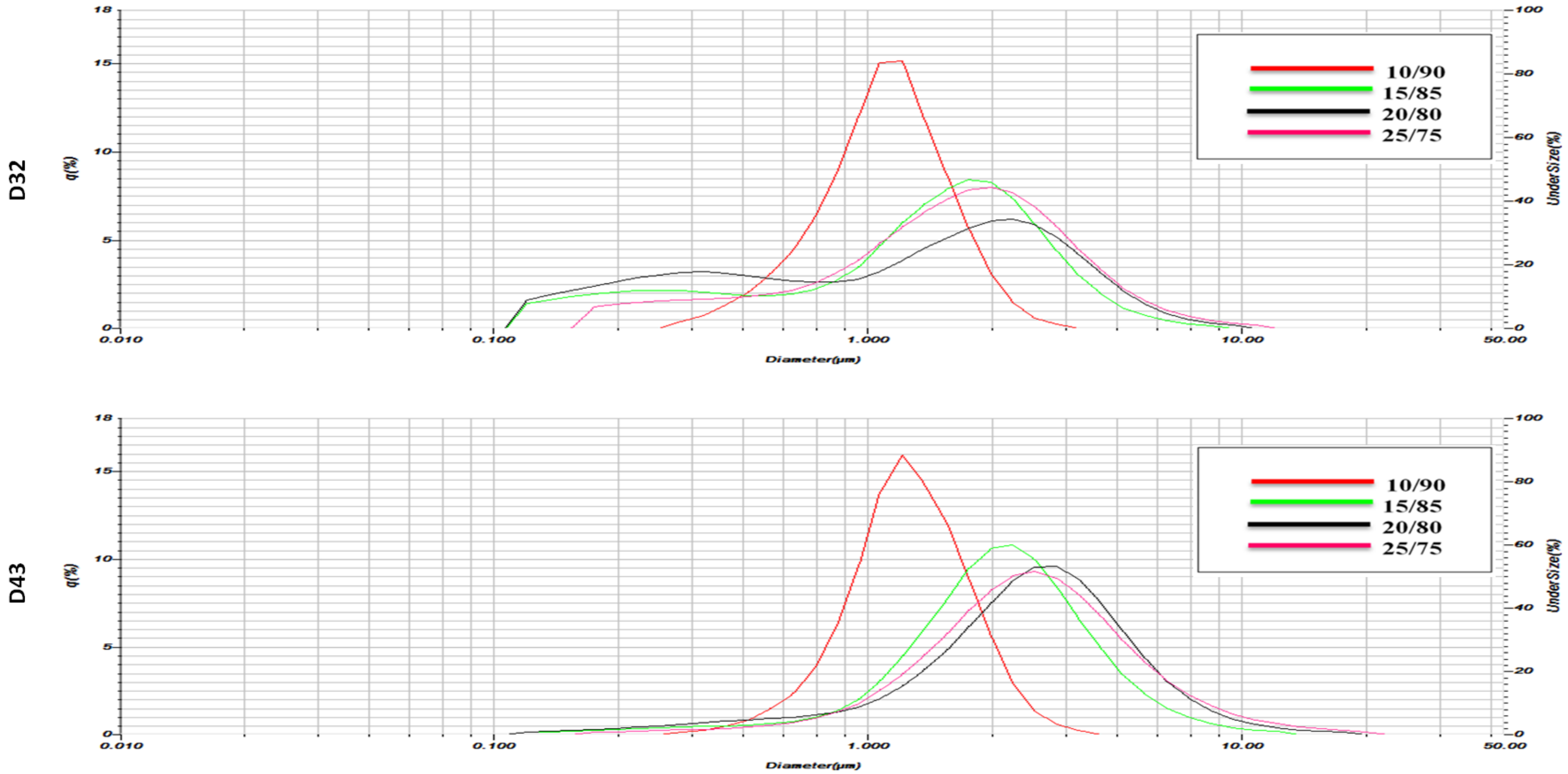

Figure 7. Particle size distribution graph of olive oil-in-bitter orange juice nano-emulsions prepared with SMP/SL with constant surfactant mixing ratio (SMP/SL= 90/10) as a function of O/S weight ratios of 10/90, 15/85, 20/80 and 25/75.

None of the emulsions prepared with SMP/SL were in nano-scale (Table 4), even though SL and SMP used in the current study have been shown to work synergistically (Compass Food Company, product specifications). Experimental results suggest that Tween80/SL and SMP/SL nano-emulsions cannot be as effective as Tween80/Span20 and Tween80/SMP nano-emulsions in preparation of olive oil-in-bitter orange juice (o/w) nano-emulsion at acidic conditions (pH 2.57).

4. CONCLUSIONS

The purpose of present study was to increase the use of bitter orange juice for flavoring and acidifying salads through raising consumer's awareness, and to promote healthy and fit life with daily diet routines. The results proved that it is possible to produce olive oil-in-bitter orange juice (o/w) nano-emulsion at acidic conditions (pH 2.57) by stabilizing binary combinations of Tween80/Span20, Tween80/SMP and Tween80/SL via phase inversion composition method. Nano-emulsions prepared with Tween80/Span20 and Tween80/SMP result with the highest transparency and relatively low particle diameters at O/S weight ratios of 15/85 and 10/90, while nano-emulsions prepared with Tween 80/SL was semi-transparent and were only in nano-scale at O/S weight ratios of 15/85 and 10/90. None of the emulsions prepared with SMP/SL were in nano-scale. Visual appearance, transparency, microstructure and particle size distribution of the nano-emulsions were influenced by surfactant blend composition and concentration. The information obtained from the current study is important for designing and commercialization of tailored olive oil-in-bitter orange juice (O/W) nano-emulsion based delivery systems with high transparency and fine droplets. For further studies, in vitro evaluation of the effectiveness of prepared nano-emulsions on weight control is suggested.

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