GRASAS Y ACEITES 72 (4)
October-December 2021, e431
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0666201

Development and characterization of ethanol-free spearmint essential oil nanoemulsion for food applications using the low energy technique

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Submitted: 16 June 2020; Accepted: 14 October 2020; Published online: 12 January 2022

SUMMARY: Different emulsifiable concentrates containing spearmint essential oil (SEO) were made and evaluated for their potential for giving ethanol-free nanoemulsion spontaneously upon dilution into water. Each one of these formulas had its specific composition regarding the type of excipients, surfactants, surfactant/SEO ratio and surfactant concentration. The results of this evaluation indicated that the chemical composition of SEO has a profound effect on the formation and physical stability of the nanoemulsion. The incorporation of excipients such as long chain triglyceride and propylene glycol into the emulsifiable concentrates at only 1.0% can lead to a stable nanoemulsion that resists Ostwald ripening. A particle size measurement showed that the diameter of SEO in the nanoemulsion was 28.2 nm and its nanostructure was maintained for 3 months. The application of a mixture of binary nonionic food-permitted surfactants enhanced the thermal stability of the nanoemulsion at up to 50 °C. The developed ethanol-free SEO nanoemulsion has promising industrial applications in food and beverage flavoring.

***KEYWORDS*:** *Essential oil; Food applications; Low energy; Nanoemulsion; Solvent-free*

RESUMEN: ***Desarrollo y caracterización de nanoemulsiones de aceite esencial de menta verde sin etanol para aplicaciones alimentarias mediante una técnica de baja energía*.** Se elaboraron y evaluaron diferentes concentrados emulsionables que contenían aceite esencial de menta verde (AMV) para determinar su potencial para dar nanoemulsión libre de etanol de forma espontánea tras la dilución en agua. Cada una de estas fórmulas tenía su composición específica en cuanto al tipo de excipientes, tensioactivos, relación tensioactivo/AMV y concentración de tensioactivo. Los resultados de esta evaluación indicaron que la composición química del AMV tiene un marcado efecto en la formación y estabilidad física de la nanoemulsión. La incorporación de excipientes como triglicéridos de cadena larga y propilenglicol en los concentrados emulsionables a solo 1,0% puede conducir a una nanoemulsión estable que resiste la maduración de Ostwald. La medida del tamaño de partícula mostró que el diámetro del AMV en la nanoemulsión era de 28,2 nm y su nanoestructura se mantuvo durante 3 meses. La aplicación de una mezcla de tensioactivos no iónicos binarios alimentariamente permitidos mejoró la estabilidad térmica de la nanoemulsión hasta 50 °C. La nanoemulsión AMV sin etanol desarrollada tiene una aplicación industrial prometedora en el sabor de alimentos y bebidas.

PALABRAS CLAVE: *Aceite esencial; Aplicaciones alimentarias; Energía baja; Libre de disolventes; Nanoemulsión*

Citation/Cómo citar este artículo: Edris AE. 2021. Development and characterization of ethanol-free spearmint essential oil nanoemulsion for food applications using the low energy technique. *Grasas Aceites* **72** (4), e431. https://doi.org/10.3989/gya.0666201

1. INTRODUCTION

Essential oils (EOs) are natural volatile extracts obtained from aromatic plants. They are frequently used as flavoring agents in food and beverages. They are also used in some cosmetic products such as fragrances, mouth washes and tooth paste due to their fragrant and antiseptic properties. Generally, EOs are highly concentrated extracts so they must be fairly diluted before application. Water, which is the most appreciated and economically feasible solvent, is inappropriate for the dilution or delivery of EOs due to their hydrophobic nature. Therefore, EOs are usually diluted and delivered using large amounts of organic solvents like ethanol and triacetin. Nowadays, the application of such solvents in food and beverages may not be appreciated by different sectors of consumers. Therefore, the emulsification of EOs in water to form solvent-free flavor delivery systems has become a promising trend for the food and beverage industry (Given, 2009). In such emulsions, EOs exist as tiny microscopic particles dispersed homogenously in water using the appropriate surfactant. However, such EO emulsions are kinetically stable which means that they can stay physically stable only for a limited period of time. Afterwards the oil droplets will flocculate, coalesce and finally separate from the system.

One of the trends that can be adopted to prolong the period of the physical stability of EO emulsions is by reducing the particle size of the dispersed EO droplets from the micrometer (1-5 μm) to the nanometer (20 nm-200 nm) size range (McClements and Jafari, 2018). The light mass of EO nanoparticles makes them more respondent to the bombardment from all directions by the molecules of the aqueous continuous phase in phenomena known as the Brownian motion (Mason *et al*., 2006). As a result, EO nanoparticles are kept in a state of continuous internal agitation which prevents their flocculation and subsequent coalescence and separation. That in return can increase the period of physical stability of the EO nanoemulsions compared to the same formula fabricated in the form of traditional macroemulsion with larger particle size (1-5 μm). Based on that principle, EO nanoemulsions were formulated for applications in food and beverage flavoring (Given, 2009; Barzegar *et al*., 2018) and for food preservation (Mazarei, and Rafati, 2019; Chu *et al*., 2020; Liew *et al*., 2020).

Two major approaches can be applied for the fabrication of EO nanoemultions, including the high energy and the low energy methods (McClements and Jafari, 2018). In the high energy method, a shear or cavitation forces are introduced into the system via high energy equipment to disrupt the EO phase into ultra-fine droplets in a continuous aqueous phase. Examples include sonicators (Rostamia *et al*., 2018), high pressure homogenizers (Martin-Piñero *et al*., 2019) and microfluidizers (Llinares *et al*., 2018). On the other hand, the low energy methods depend on the handling of the interfacial properties of the system by managing the appropriate type and ratio of excipients and surfactants used in the formulation process (Komaiko and McClements, 2016). Therefore, the low energy method does not rely on high shear equipment for the fabrication of nanoemulsions. The low energy method can possibly be carried out by three different approaches, namely spontaneous emulsification (Yildirim *et al*., 2017), phase inversion temperature (Chuesiang *et al*., 2018), and phase inversion composition (Chu *et al*., 2020). Each of these methods has its own preparation steps, advantages and liability which were conclusively reviewed elsewhere (Komaiko and McClements, 2016).

In the current investigation, the author aimed to develop ethanol-free and physically stable spearmint essential oil (SEO) nanoemulsion. This essential oil was chosen due to its promising potentials as a natural flavoring agent in innovative beverages like soft drinks and flavored water. The oil also has antimicrobial activity against some food related fungi (Ji *et al*., 2019) and pathogenic bacteria (Snoussi *et al*., 2015). All of the previously mentioned potentials of SEO in beverages and food necessitate the development of a water-based colloidal system like nanoemulsion for the delivery of SEO in these applications.

It is important to point out that there were previous investigators who reported the formulation of SEO nanoemulsion. However, they used high temperature (65 °C) along with high energy equipment (Tubtimsri *et al*., 2018) or large amounts of ethanol in their formulations (Wangjit *et al*., 2016).

Based on that, the current investigation was devoted to the development and characterization of ethanol-free SEO nanoemulsion fabricated at room temperature. The low energy spontaneous emulsification method is adopted for that purpose due to its simplicity, non-destructive property on the structure of EO (as it takes place at room temperature), and economic feasibility to scale up to industrial level. The appropriate measurements which are necessary to characterize the development of nanoemulsion were carried out. Critical issues that can radically influence the formulation and thermal stability of ethanol-free SEO nanoemulsions were also investigated and discussed in detail.

2. MATERIALS AND METHODS

2.1. Materials

Spearmint essential oil (SEO) was obtained from a freshly distilled batch produced at the Horticultural Research Institute, Medicinal and Aromatic Plant Research Section, Kanater, Egypt. The oil was obtained from the aerial parts (leaf and stacks) of *Mentha spicata*, Family La-

Table 1. Composition of the different sets of spearmint essential oil emulsifiable concentrates.

Sets of SEO	Surfactant(s) and their weight ratios	Composition of the final nanoemulsion (weight %)[a]					
		Sufactant(s)	Essential oils (SEO)	Ripening inhibitor		Co-surfactant (PG)	S/O ratio
				(LCT)	(MCT)		
Set (I)							
I(a)	T80	6	6	---	---	---	1
I(b)	T80	6	5	1	---	---	1
I(c)	T80	6	5	---	1	---	1
I(d)	T80	6	5	1	---	1	1
I(e)	T80	3	5	1	---	1	0.5
Set (II)							
II(a)	T80-T20 (2:1)	6	5	1	---	1	1
II(b)	T80-T20 (1:1)	6	5	1	---	1	1
II(c)	T80-T20 (1:2)	6	5	1	---	1	1
Set (III)							
III (a)	T80-Cr (1:1)	6	5	1	---	1	1
III (b)	T80-Cr (2:1)	6	5	1	---	1	1
III (c)	T80-Cr (1:2)	6	5	1	---	1	1

[a]Each emulsifiable concentrate of the three sets is added to the amount of water sufficient to give 100% final SEO nanoemulsion.
SEO: spearmint essential oil; T80: Tween 80; T20: Tween 20; Cr: Cremophor RH40.
LCT: Long-chain triglyceride; MCT: médium-chain triglyceride.
S/O ratio: surfactant / oil ratio; PG: propylene glycol.
Samples are made in duplicate.

miaceae, after steam distillation, as indicated by the supplier. After distillation, SEO was kept at – 4 °C in dark glass bottles with no headspace till analysis and formulation. Tween 80, Cremophor (Kolliphor) RH40®, propylene glycol, *l*-carvone, *d*-limonene, 1,8-cineol and pulegone were purchased from Sigma-Aldrich (St. Louis Missouri, USA). Sunflower oil, which was used as a source of long chain triglyceride (LCT), was purchased from the local market. Labrafac™ lipophile WL 1349, which is a medium-chain triglyceride (MCT) composed of C_8 and C_{10} fatty acids, was donated from Gattefosse, France.

2.2. Determination of the chemical composition of SEO

SEO was subjected to gas chromatographic (GC) analysis to reveal the structure of its major volatile constituents. For that purpose, SEO (20 µL) was diluted in 1 mL diethyl ether in a glass vial. Then, 2.0 µL of this mixture were injected (at a split ratio 10:1) into Agilent GC equipped with a flame ionization detector (FID). A fused silica capillary column DB5 (30 m × 0.32 mm × 0.25 µm) was used to separate the different volatile components. The oven temperature was programmed from 50 °C to 220 °C at a rate of 3 °C/min. The injector and detector temperatures were 220 °C and 230 °C, respectively. Helium was used as carrier gas at a flow rate of 1 mL/min. Standard samples of *l*-carvone and *d*-limonene, 1,8-cineol and pulegone were used to identify their corresponding equivalents in SEO. The volatile oil constituents were reported as percent of the total eluted peak areas after FID. All values were means of two injections ±S.D.

2.3. Calculation of the HLB values of the binary surfactant systems

The HLB (the hydrophilic lipophylic balance) of a binary surfactant mixture made of (Tween 80/Tween 20) at different ratios was mathematically calculated using the original formula (Strianse and Lanzet, 1960):

$$HLB_{mix} = (\Phi_A \times HLB_A) + (1- \Phi_A \times HLB_B)$$

Where, Φ_A: Mass fraction of the first surfactant; HLB_A: HLB of the first surfactant; (1- Φ_A): Mass fraction of the second surfactant; HLB_B: HLB of the second surfactant

2.4. Formulation of SEO nanoemulsions

Three principle sets of SEO emulsifiable concentrates which included 11 different formulas were made at specific composition and weight ratios as described in Table 1. The desired SEO nanoemulsions were fabricated from the emulsifiable concentrates using the low-energy spontaneous emulsification method as previously described (El-Sayed *et al*., 2017), from the original work of Anton and Vandamme (Anton and Vandamme, 2009). In more detail, the formulation process was initiated by the preparation of three SEO emulsifiable concentrates which were

made as follows: SEO was first mixed with MCT or LCT to form a clear isotropic mixed oil phase; then, the later was added to the single surfactant (Set I) or the binary surfactant mix (Set II & III) in the presence or absence of PG as a cosurfactant (Table 1); the whole mixture was then vortexed to end up with different SEO emulsifiable concentrates. It was possible to heat the concentrates up to 40 °C using water bath in order to decrease the viscosity of the sets to intimate the mixing of ingredients during a vortex application.

Finally, after 30 minutes of equilibration at room temperature, each set of SEO emulsifiable concentrates was titrated drop-wise to a calculated amount of distilled water which was sufficient to produce nanoemulsions containing 6.0% of total oil phase, as illustrated in Table 1. During titration, water was subjected to continuous agitation using a magnetic bar till the whole load of each SEO concentrate was titrated. The samples were then transferred to transparent glass vials and stored at room temperature (~ 20 °C) for further analysis. All formulations were made in duplicate.

2.5. Characterization of SEO nanoemulsions

2.5.1. Appearance

Glass vials containing the different SEO nanoemulsions were examined visually against bright light to check their opacity or translucency. The UV-Vis absorption values (A) for all samples were also evaluated spectrophotometrically using Shimadzu (UV-160 1PC spectrophotometer, Japan) at wave length = 600 nm to establish a quantitative measure for the visual examination of transparency of the samples. The absorption values (A) are an average of two measurements from two different formulations.

2.5.2. Particle size

The particle size of the samples was measured using the dynamic light scattering instrument Zetasizer (Nano-ZS model ZEN3600, Nanoseries, Malvern Instruments, UK). Measurements were taken at 20 ºC unless otherwise specified, with a fixed angle of 173º. Before measurement, all samples were filtered through 0.20 µm single-use syringe filter unit (Minisart®, Sartoius Stedium Biotech GmbH Germany) to remove impurities. Each sample was diluted before measurement with distilled water to only 0.05% to prevent multiple scattering. The measurements are based on the Brownian motion of the hydrated particles thus; it provides information on the hydrodynamic diameter (nm) of the SEO particles. The sizes quoted are the *z*-average mean of the SEO droplet's hydrodynamic diameter (nm) obtained from 6 measurements for each sample (2 duplicate x 3 measurements each) ±SD, as shown in Table (2). The particle size distribution curves are plotted from the dynamic laser scattering results from duplicate samples.

2.6. Evaluation of storage stability

The different formulas of SEO nanoemulsions were subjected to a storage stability test by being left to stand undisturbed on the bench at room temperature (20 ºC ±2) for 3 months. In addition, another storage stability test at

Table 2. HLB values and particle size analysis of the different SEO nanoemulsions formulated and stored at different temperatures.

Sets of SEO	HLB of surfactant(s)	Particle size (nm) and polydispersibility index (PDI)			
		Zero time at 20 ºC	3-month storage at 20 ºC	5-days storage at 40 ºC	5-days storage at 50 ºC
Set (I)					
I(a)	15	121.3±1.7 (PDI 0.42)	Creamy layer after 24h	Cloudy after 24h	NM
I(b)	15	38.0 ±0.5 (PDI 0.30)	35.0±0.2 (PDI 0.2)	Cloudy after 24h	NM
I(c)	15	96.3 ±0.8 (PDI 0.41)	Creamy layer after 24h	Cloudy after 24h	NM
I(d)	15	28.2 ±0.1 (PDI 0.19)	25.9±0.1 (PDI 0.1)	Cloudy after 24h	NM
I(e)	15	62.4 ±0.2 (PDI 0.18)	71.9±0.7 (PDI 0.3)	Cloudy after 24h	NM
Set (II)					
II(a)	15.55	29.5±0.2 (PDI 0.2)	NM	Cloudy after 24h	NM
II(b)	15.85	69.2±2.0 (PDI 0.5)	NM	Cloudy after 24h	NM
II(c)	16.11	106.9±0.6 (PDI 0.2)	NM	Cloudy after 24h	NM
Set (III)					
III (a)	NC	25.4±0.1 (PDI 0.1)	NM	26.7±0.2 (PDI 0.1)	49.1±0.1 (PDI 0.2)
III (b)	NC	24.4±0.1 (PDI 0.1)	NM	44.7±1.4 (PDI 0.2)	75.5±0.2 (PDI 0.1)
III (c)	NC	32.0±0.3 (PDI 0.2)	NM	NM	NM

HLB: hydrophilic lipophilic balance; NC: Not calculated because there is no specific HLB value for Cremophor RH 40.
NM: Not measured because of the physical instability manifested by the cloudy appearance; Samples were made in duplicate.

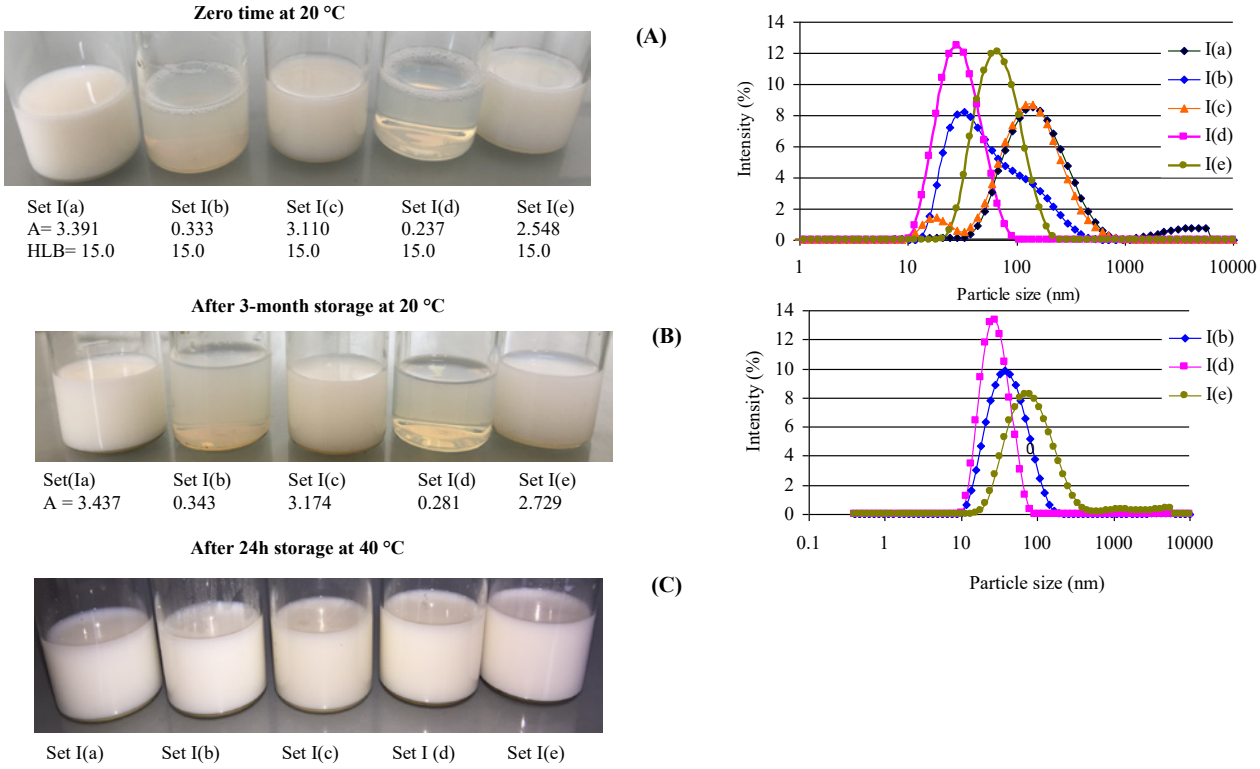

FIGURE 1. Appearance, UV-Vis absorption "A" at 600 nm, (left column) and their corresponding particle size analysis (right column) of the different spearmint essential oil nanoemulsions fabricated from set (I) using single surfactant at different storage periods and temperatures. The absorption values (A) are an average of two measurements from two different formulations, SDs are too small (≤0.005) to be indicated. The particle size distributions are an average of two measurements from two different formulations, plotted from dynamic laser scattering results.

higher temperatures was performed by incubating the formulas (undisturbed) at 40 ºC and 50 ºC ±1 for 5 consecutive days. At the end of the storage period, the formulas were visually inspected to detect any change in appearance, which was also quantified as absorbance values (A) using UV-Viz examination at 600 nm. Finally, the particle size of the stored samples was re-analyzed for potential alteration during storage as previously described in section (2.5.2).

2.7. Statistical analyses

Data reported after the gas chromatographic analysis of SEO and also after the particle size measurements of the nanoemulsions were the means of at least 2 replicates ± S.D.

3. RESULTS

3.1. Chemical composition of spearmint essential oil (SEO)

The composition of SEO was revealed using the gas chromatographic analysis (GC). The results indicated the presence of high contents in *l*-carvone (63.3±0.9%), and *d*-limonene (12.4±0.3%) besides much lower contents in 1,8-cineol (5.6±0.1%) and pulegone (3.6±0.1%). All together, these components accounted for about 85.0% of the oil composition, which came within the average percentages that were previously reported for each one of these components in SEO (Edris *et al*., 2003; Bouyahya *et al*., 2017; Snoussi *et al*., 2015). No attempts were made to identify the rest of the components because the characterized components were sufficient for the purpose of the GC analysis.

3.2. Formulation and characterization of SEO nanoemulsion of Set (I)

3.2.1. The effect of triglyceride

In a trial to produce ethanol-free nanoemulsion upon dilution into water, a basic tertiary emulsifiable concentrate (Ia) composed of SEO (6.0%), single surfactant (6.0%), water (88.0%) was made (Table 1). No traces of triglyceride oil were used in this formula to see if the desired nanoemulsion could be developed using SEO as the sole oil phase. The results showed that upon dilution of (Ia) emulsifiable concentrate into water, a traditional cloudy emulsion which had high UV-Vis absorbance (A= 3.39, Figure 1A) was obtained. That indicated a failure

in the formulation of the desired nanoemulsion. Although the average particle size of that emulsion was 121.3 nm (Table 2), its size distribution analysis showed a second population of particles with an average size of about 5.0 μm (Figure 1A**,** chart). That makes the emulsion of (Ia) appear cloudier due to enhanced light scattering by the large particle population which also caused a relatively high polydispersibility index (Table 2).

Regarding physical stability, emulsion (Ia) demonstrated poor stability as evident from the appearance of a heavy creamy layer on the walls of the glass vial upon tilting to 45º within only 24h of storage at room temperature (20 °C). These results are likely to be due to the phenomena of Ostwald ripening, (OR).

The incorporation of 1.0 wt% long-chain triglyceride (LCT), such as sunflower oil (set Ib, Table 1) led to the inhibition of OR and positively influenced the appearance, particle size, and shelf-life stability of the obtained SEO nanoemulsion, (Figure 1A and Table 2). This was manifested by a change in UV-Vis absorption of the nanoemulsion of (Ib) to become 0.33, which was in accordance with its less cloudy appearance compared to (Ia), as shown in Figure 1A. In addition, the nanoemulsion of set (Ib) was characterized by a much smaller particle size (38.0±0.5 nm) with better size distribution and lower PDI, as evident from the data in Table 2 and Figure 1A (chart). Unlike the emulsion of set (Ia), which creamed after 24h, the new nanoemulsion of (Ib) which contained LCT overcame OR and showed much higher physical stability. It maintained the same appearance with almost the same UV-Vis absorption even after the 3-month storage period at 20 ºC (Figure 1B). The particle size of nanoemulsion (Ib) after the end of the 3-month storage was 35.0±0.2 nm (Table 2) and its size distribution was monomodal (Figure 1B**,** chart). The particle size of (Ib) and its distribution pattern were even better after storage than at time zero. The reason behind this result is that storing (Ib) for 3months provided enough time for SEO nanoparticles to equilibrate and attain their final size. That chance was not offered to the same formula at time zero because particle sizing took place shortly after fabrication.

The reader may notice that the particle sizing of emulsions (Ia) and (Ic) are excluded from analysis after the 3-month storage period and seemed absent from Figure 1B **(**chart). This is because they initially showed early creaming after 24h (Ia), and 1 week (Ic), respectively, as will be shown in the results regarding nanoemulsion of set (Ic).

Despite the high physical stability of the nanoemulsion of (Ib) at 20 ºC, it showed temperature sensitivity when stored at 40 ºC for only 24h. That was manifested by the conversion of its appearance into milky white (Figure 1C), indicating an increase in particle size and loss of the nanostructure.

The incorporation of 1.0 wt% of another triglyceride oil with medium-chain fatty acids (MCT) such as C_8 and C_{10} (set Ic, Table 1) also led to the inhibition of OR. However, the quality of the obtained emulsion of (Ic) was inferior compared to the nanoemulsion of (Ib) which was formulated using LCT. For instance, Figure 1A indicates that the appearance of nanoemulsion (Ic) was cloudy with a much higher UV-Vis absorbance value compared to (Ib). The particle size of (Ic) was 96.3±0.8 nm which represents more than double the size of nanoemulsion (Ib), as shown in Table 2. The particle size distribution of (Ic) also showed a bimodal pattern which justified its relatively high PDI (0.41, Table 2). The shelf-life stability of emulsion (Ic) was also inferior compared to (Ib). After only 1 week of storage at room temperature, the nanoemulsion of (Ic) showed a thin creamy layer on the side of the glass vials upon tilting to 45º. Regarding thermal stability, the emulsion of (Ic) became even cloudier after storage for 24h at 40 ºC (Figure 1C).

3.2.2. The effect of cosurfactant

An additional emulsifiable concentrate (Id) was formulated with the incorporation of 1.0 wt% propylene glycol (PG) as a cosurfactant (Table 1). Results of the effect of PG on the formation and stability of SEO nanoemulsion showed that PG increased the transparency of the nanoemulsion (Id) to become the clearest among those of set (I), (Figure 1A). The average particle size of that nanoemulsion was 28.2±0.1 nm which is considered to be the smallest among all nanoemulsions of set (I) as shown in (Table 2). This result demonstrates the potential of PG at only 1.0 wt% of the formula to act as an efficient cosurfactant that can replace ethanol for enhancing the formulation of SEO nanoemulsion with an ultra-fine particle size.

The helf-life stability study indicated that the nanoemulsion which contained PG was physically stable for 3(+) months as long as the temperature was ≤ 20 ºC. No noticeable change in transparency or in the average particle size after storage (25.9±0.1 nm) was detected as shown in Table 2, and Figure (1B, chart). It is worth noting that these results cannot be attained with PG concentration < 1.0%, as revealed in a preliminary experiment in the current study (data not shown).

Unfortunately, despite the distinctive effect of PG on the formulation and stability of SEO nanoemulsion of (Id), the formula showed temperature sensitivity when stored at 40 ºC for only 24h. That was manifested by a radical change in appearance from the most translucent nanoemulsion into milky white macroemulsion (Figure 1C). That indicates a dramatic increase in particle size and loss of nanostructure.

3.2.3. The effect of surfactant/oil ratio (S/O ratio)

A fifth emulsifiable concentrate (Ie) was made among the formulas of set (I) using the ingredients of formula (Id) after reducing its S/O ratio from 1.0 to 0.5 (Table 1).

The effect of surfactant reduction on the stability of the ethanol-free SEO nanoemulsion was studies. The results in Figure 1A show that the obtained nanoemulsion of (Ie) had an opaque appearance with a monomodal particle size distribution. The average particle size was 62.4±0.2 nm (Table 2), which is about double the size of the nanoemulsion of (Id) in which the S/O ratio was higher (equals 1.0). After a 3-month storage period at 20 °C the particle size of (Ie) slightly changed from 62.4 nm to 71.9±0.7 nm (Table 2), with monomodal size distribution as shown in Figure 1B, (chart).

There was also no detected separation or formation of creamy layer after the 3-month storage period. This result is considered to be satisfactory for a stable nanoemulsion because the particle size remained < 100 nm after storage, with no signs of creaming. The reader can also take into consideration that this result was obtained at 50.0% less surfactant with respect to the most ideal physically stable nanoemulsion of (Id).

Subjecting the nanoemulsion of (Ie) thermal stress by storage at 40 ºC led to the conversion of the nanoemulsion into a milky white macroemulsion after only 24h (Figure 1C).

3.3. The effect of HLB on the formation and thermal stability of SEO nanoemulsions

A mixture of binary surfactants composed of Tween 80 and Tween 20 at three different weight ratios was used to formulate a second set of SEO nanoemulsions (set II, Table 1). This mixture of surfactants offers different HLB (hydrophilic lipophylic balance) values, which can affect the formation and thermal stability of SEO nanoemulsion. The results illustrated in Figure 2A and Table 2 reveal that the nanoemulsion of set (IIa) had a translucent appearance and monomodal size distribution pattern accompanied by ultra-fine particle size (29.5±0.2 nm). On the other hand, nanoemulsions of (IIb and IIc) had much larger particle size (69.2±2.0 nm and 106.9±0.6 nm, respectively). This means that the HLB value for formula IIa (15.55, Table 2), which corresponds to the surfactant mixture composed of (T80-T20), at 2:1, is more suitable for the formation of SEO naoemulsion than the HLB values for the other surfactant ratios of II(b) and II(c), (Table 2).

The three nanoemulsions (IIa-c) were subjected to a mild thermal stress by storing at 40 °C. The results showed that this treatment led to destabilization, as evident from the change in the appearance of all nanoemulsions of set (II) to milky white after only 24h (Figure 2B).

Based on the previously obtained results, a second trial was performed to enhance the thermal stability of the SEO nanoemulsion, by formulating a third set of emulsifiable concentrate (set III, Table 1). This set was made using a different surfactant mixture made of Tween 80 and Cremophor RH40, which is another food-permitted surfactant.

The results from that approach indicated that the nanoemulsions of (IIIa) and (IIIb) had excellent transparent/

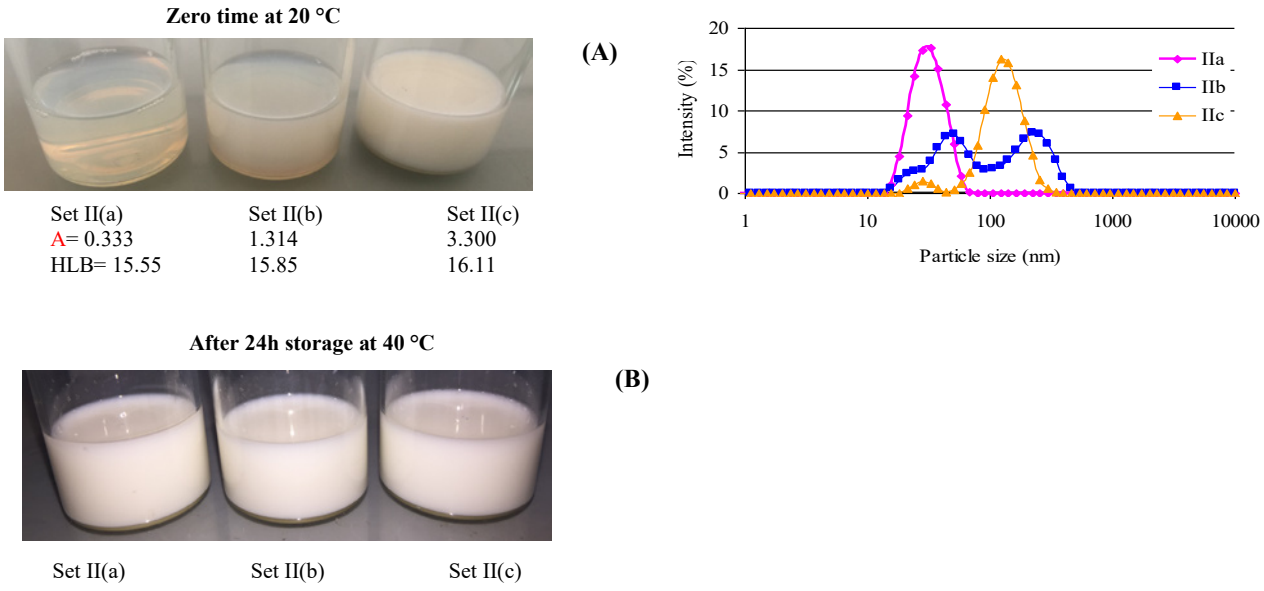

FIGURE 2. Appearance, UV-Vis absorption "A" at 600 nm, HLB values, (left column) and their corresponding particle size analysis (right column) of the different spearmint essential oil nanoemulsions fabricated from set (II) using family binary surfactant mix at different storage periods and temperatures.
HLB: hydrophilic lipophilic balance
The absorption values (A) are an average of two measurements from two different formulations, SDs are too small (≤0.005) to be indicated.
The particle size distributions are an average of two measurements from two different formulations, plotted from dynamic laser scattering results.

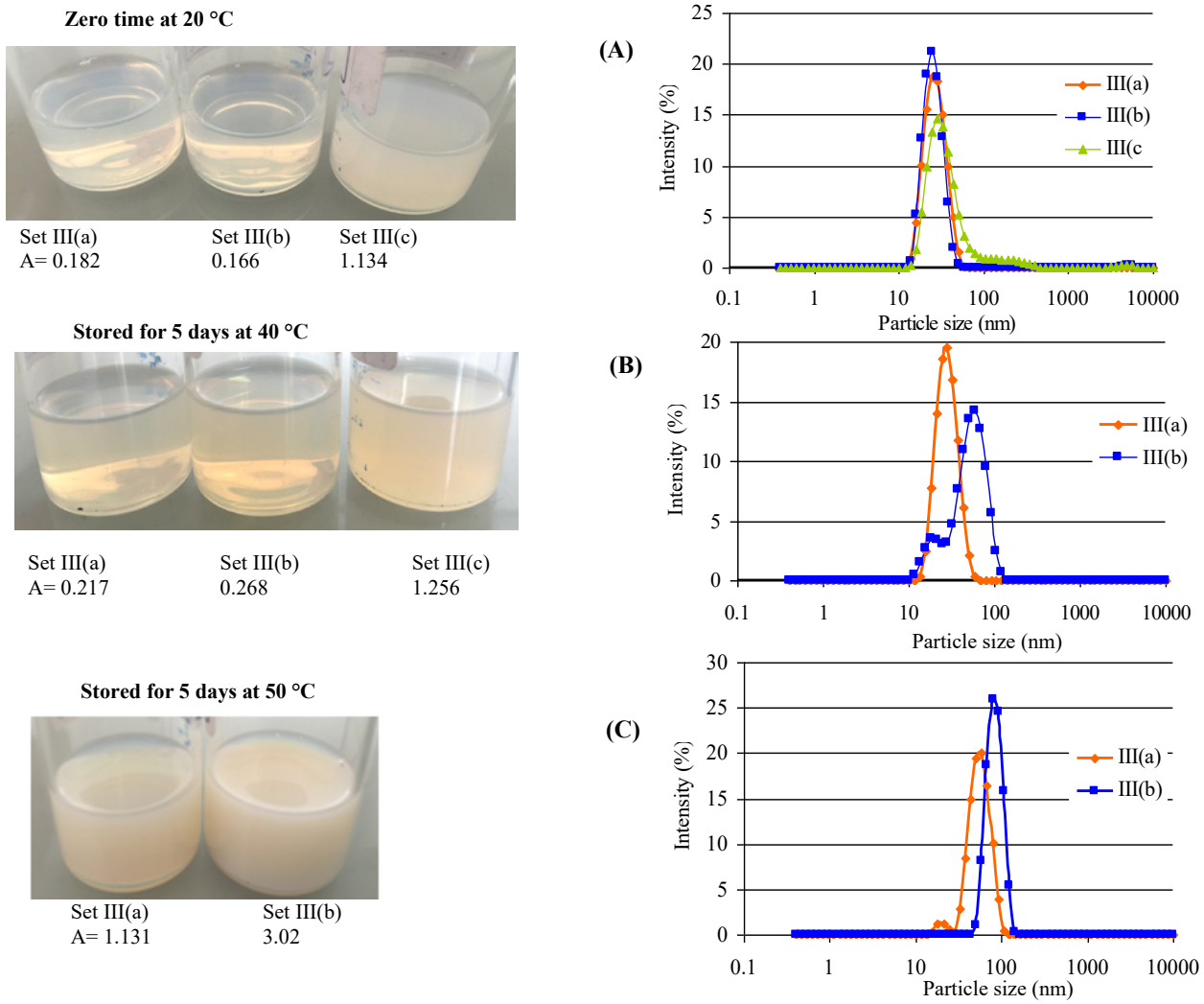

FIGURE 3. Appearance, UV-Vis absorption "A" at 600 nm, (left column) and their corresponding particle size analysis (right column) of the different spearmint essential oil nanoemulsions fabricated from set (III) using hetero binary surfactant mix at different storage periods and temperatures. The absorption values (A) are an average of two measurements from two different formulations, SDs are too small (≤0.005) to be indicated. The particle size distributions are an average of two measurements from two different formulations, plotted from dynamic laser scattering results.

translucent appearance (Figure 3A) with very small particle size (25.4±0.1 nm and 24.4±0.1 nm, respectively), as shown in Figure (3A, chart), and Table (2). On the other hand, the nanoemulsion of set (IIIc) was more opaque with higher absorbance in UV-Vis. Despite the small average particle size of this nanoemulsion (32.0±0.3 nm), its size distribution showed a second small population of larger particles with average particle size 114.0 nm (Figure 3A**,** chart), Which may have been responsible for the opaque appearance of (IIIc).

Subjecting all nanoemulsions of set (III) to a thermal stability evaluation by storing at 40 °C for 5 days indicated that only slight changes were observed in visual appearance and in the change in the UV-Vis absorbance of the three nanoemulsions, (Figure 3B). Interestingly, none of these samples turned milky white as was the case with nanoemulsions of sets (I and II), even after 5 days of storage at 40 °C. The particle size of SEO nanoemulsion of set (IIIa) after the thermal treatment was 26.7±0.2 nm, which was almost the same as before the thermal treatment (25.4±0.0 nm, Table 2). On the other hand, the size of (IIIb) increased by about double after the thermal storage to become 44.7±1.4 nm. The nanoemulsion of set (IIIc) was excluded from particle sizing due to its cloudier appearance after the thermal treatment at 40 ºC compared to (IIIa,b), indicating less thermal tolerance (Figure 3B).

From these promising results it can be concluded that SEO nanoemulsions of set (IIIa) and (IIIb) are more thermally stable and temperature tolerant up to 40 °C compared to all nanoemulsions produced using sets I and II.

Therefore, the same nanoemulsions were further challenged with a second round of higher temperature by storing at 50 °C for another 5 days. The results from this treatment showed that the transparency of the nanoemulsions of set (IIIa) and (IIIb) decreased but did not become totally milky white (Figure 3C). The particle size after the second thermal treatment (at 50 °C) was 49.1±0.1 and 75.5±0.2 nm, respectively, (Table 2). In addition, their PDI was 0.149 and 0.114, respectively, indicating a uniform and narrow particle size distribution (Figure 3C**,** chart).

4. DISCUSSION

4.1. Chemical composition of SEO

A GC analysis of SEO was established for two main reasons: first, to confirm the authenticity of the supplied oil through the characterization of its major volatile constituents; and second, to justify the stability behavior of the developed ethanol-free SEO nanoemulsions based on examination of its main constituents.

As shown in the results section, the major constituents of SEO were *l*-carvone, *d*-limonene with some other lower contents of 1,8-cineol and pulegone. This result can authenticate the oil sample in the current study as *Mentha spicata,* which is precisely the targeted oil. It is important to note that there are other species of mint which may also be used in the food industry, such as *Mentha piperita* and *Mentha pulegium*. These species are characterized by different chemical compositions which are rich in menthol and pulegone, respectively.

Therefore, they can show different behavior in the formation of nanoemulsion compared to *Mentha spicata,* which was used in the current study.

4.2. Formulation and characterization of SEO nanoemulsion of Set (I)

Set (I, Table 1) is a group of five emulsifiable concentrates which were formulated to examine their potential to give ethanol-free SEO nanoemulsion spontaneously upon dilution into water. This set was formulated using Tween 80 as a sole surfactant in the absence or presence of other food-grade excipients. These excipients include long-chain triglycerides (LCT) as in formula (Ib) and médium-chain triglycerides (MCT) as in (Ic). Both triglycerides are known as Ostwald ripening inhibitors (ORI) which are commonly used to stabilize EO nanoemulsions. Beside the triglycerides, a third excipient, namely propylene glycol (PG) was also incorporated along with the ORI to replace ethanol as another food-permitted cosurfactant (formula Id). The surfactant to oil ratio (S/O ratio) in the previous four formulas (Ia - Id) was kept at 1.0, which means that the mass of surfactant equals that of SEO, (Table 1). This relatively high ratio (i.e. high amount of surfactant) was used because it is usually applied in the low energy emulsification method to compensate for the absence of high shear energy equipment (Anton, and Vandamme, 2009). Beside formulas (Ia-Id), a fifth formula (Ie) was fabricated using the same excipients as in (Id) after reducing the amount of surfactant by 50.0% so that the S/O ratio became only 0.5 (Table 1).

The choice of the constituents for the previously mentioned five emulsifiable concentrates (Ia to Ie) of set (I) was meant to be a pattern for evaluating the effect of surfactant type, excipients, and the S/O ratio on the formulation and physical stability of the ethanol-free nanoemulsions at room temperature and also under thermal challenging conditions. Therefore, parameters like appearance, UV-Vis absorption at 600 nm, particle size measurement, and the change of these parameters after storage at higher temperatures were assessed and taken as indicators in this evaluation. The effect of each one of these parameters on nanoemulsion formation and stability is discussed in details in the following sections.

4.2.1. The effect of triglyceride

Formula (Ia), which contained no traces of triglycerides (Table 1), failed to produce SEO nanoemulsion and showed poor physical stability in less than 24h. One should bear in mind that the S/O ratio of emulsion (Ia) equals 1.0. That means the presence of a high amount of surfactant, enough to support the formation of a stable emulsion even without using high energy shear equipment in the emulsification process. Therefore, there should be a factor which caused such rapid creaming in this formula which is likely to be the phenomena of Ostwald ripening (OR), (McClements *et al*., 2012). OR occurs in nanoemulsions (or emulsions) when the oil phase is slightly soluble in water, such as the case with essential oils. OR takes place as a result of the growth of large oil droplets at the expense of tiny ones. That happens due to the continuous migration (through water) of oil from the tiny droplet to the larger droplets under the effect of the high curvature pressure "Laplace pressure" which is exerted on the tiny droplets. Therefore, large droplets become larger and finally float to the surface causing a creamy layer in a short period of time, just a few hours after formulation (McClements *et al*., 2012).

EO emulsions and nanoemulsions are a typical example of the manifestation of OR due to the slight water solubility of their ingredients (McClements *et al*., 2012). This was clearly proven in the current study in the case of formula (Ia) due to the slight water solubility of the major volatile constituents of SEO. For instance, the water solubility of *l*-carvone, *d*-limonene and 1,8-cineol, which are the three major constituents of SEO, was 27.0 $mg \cdot l^{-1}$, 13.8 $mg \cdot l^{-1}$ and 552.0 $mg \cdot l^{-1}$, respectively, as indicated by the official database (PPDB & BPDB, web sites). These three constituents together represented about 81.2% of the total

composition of SEO, as was revealed in the current study by GC analysis. Therefore, the author concludes that OR contributes significantly to the observed early creaming of formula (Ia).

In a trial to inhibit OR and to develop more stable SEO nanoemulsion, we incorporated equal amounts (1.0wt %) of two different triglyceride oils into formula (Ib) and (Ic). The first was sunflower oil, which is considered to have long-chain triglycerides (LCT, set Ib), while the second was synthetic medium-chain triglycerides (MCT) made of C_8-C_{10} (set Ic), as shown in Table 1.

The entropy of mixing of SEO with these triglyceride oils provided chemical potential against OR and counterbalanced the driving force for EO migration from tiny particles to large ones (McClements *et al*., 2012).

This treatment showed a noticeable improvement in the appearance, particle size and storage stability of the SEO nanoemulsion, as shown in the results section (Table 2) and (Figure 1A, B). However, it was clear from the results that LCT was a much better excipient than MCT in the formulation of stable SEO nanoemulsion with respect to two aspects: first, better inhibition of OR; and second, greater influence on the reduction of SEO particle size. Detailed justifications of these two findings are discussed in the next passage.

First, the ability of LCT to inhibit OR to a better degree compared to MCT is due to its strong hydrophobic nature (HLB 4.0) compared to that of MCT (HLB 8.0), (Wooster *et al*., 2008). The strong hydrophobicity of LCT originates from its long chain fatty acids which include linoleic (C18:2), oleic (C18:1) and stearic (C18:0) acids. These fatty acids constitute the composition of the sunflower oil which was used as LCT. Therefore, the spiking of SEO with such a small amount (1.0 wt%) of LCT, (as in nanoemulsion of set Ib) can reduce the water solubility of its volatile ingredients to a great extent compared to MCT, leading to better inhibition of OR. That conclusion came in accordance with another study (McClements *et al*., 2012) which further concluded that the hydrocarbon chain length of the triglyceride can affect the interfacial tension between EO droplets and water, and consequently, affect the overall nanoemulsion stability. It is worth indicating that MCT is composed of medium-chain caprylic (C_8) and capric (C_{10}) acids which are less hydrophobic than the other long-chain fatty acids.

On the other hand, our second finding concerning the greater influence of LCT on the reduction of the particle size of SEO nanoemulsion of set (Ib) compared to (Ic) can be justified based on the physical affinity between the oil phase mixture (SEO/triglyceride) and the utilized surfactant. The high affinity between these ingredients leads to a high tendency toward the production of nanoemulsion with ultra-fine particle size (Anton and Vandamme, 2009). Our results indicated that the affinity between (SEO/LCT) and Tween 80, which is used as a surfactant, is higher than that of (SEO/MCT) and the same surfactant. This conclusion is based on the ability of (SEO/LCT) to produce nanoemulsions (Ib) with smaller particle size (38.0 nm) compared to (Ic, 96.0 nm) which was made of (SEO/MCT), as shown in Table 2.

4.2.2. The effect of cosurfactant

Ethanol is usually used as cosurfactant in a relatively high amount to facilitate the formulation of EO nanoemulsions. It can be left in the nanomemulsion after fabrication (Zhang *et al*., 2017), or removed by evaporation in a process known as solvent displacement (Katata *et al*., 2017). However, the presence of ethanol in food and beverages is met with objection from some consumers who seek solvent-free products.

Therefore, out of the desire to eliminate ethanol from food or beverages, we replaced ethanol with only 1.0 wt% PG as cosurfactant. PG is a food-permitted ingredient which has a wide safety margin that can reach up to 25mg/Kg body wt./day (Joint FAO/WHO, 2002). Therefore, another emulsifiable concentrate (set Id, Table 1) was developed based on the ingredients of the promising formula (Ib) with the incorporation of 1.0 wt% PG as cosurfactant (Table 1). As a result, a new SEO nanoemulsion (Id) with better transparent appearance (Figure 1A), smallest particle size (28.2 nm, Table 2) and high stability was obtained.

The mechanism of the action of PG as cosurfactant which is able to facilitate the formation of nanoparticles and to enhance its physical stability is similar to ethanol and other short chain alcohols (Garti *et al*., 2001). PG can arrange itself along with the main surfactant at the interfacial layer between SEO and water, leading to the formation of a mixed surfactant film. This behavior lends flexibility to the film around SEO droplets, leading to an ease in attaining the maximum curvature required for ultra-fine oil particles to be developed. In addition, PG can modify the hydrophobicity of the main surfactant, making it more compatible with the SEO phase.

It is important to note that the role of PG in the formation of ethanol-free nanoemulsion cannot be generalized to include all other types of essential oils. This statement was concluded by the author himself who found that PG does not make any improvement in the formulation of clove or cinnamon EO nanoemulsions (data not published). The particle size of these nanoemulsions increased in the presence of PG rather than decreased as expected. At this point, the real reasons behind that peculiar behavior are not known. However, it can be speculated that the chemical composition of the EO as well as its interaction with the chosen surfactant must have an effect. Further investigations are required in this respect to unravel the

scientific basis behind the effect of PG on the formation of various types of EO nanoemulsions.

4.2.3. The effect of surfactant/oil ratio (S/O ratio)

The S/O ratio is considered to be a key factor in the fabrication of EO nanoemulsions using the low energy spontaneous emulsification method. This ratio represents the product of dividing the mass of surfactant by that of the oil phase. The common ratio which is usually used in the art to induce the formation of nanoemulsion is 1.0 (i.e. equal masses of surfactant and oil). However, this ratio can be increased in the case of other types of formulations to become 2.0 (Komaiko and McClements, 2015). This means doubling the amount of surfactant relative to EO.

In the current study the S/O ratio equal to 1.0 was used in the four emulsifiable concentrates of set (I) from (Ia-Id). However, a reduction in the amount of surfactant is generally appreciated in order to fulfill the requirements of legislation in food and beverages and also to proceed economically. However, that trend is considered to be a challenge for formulators due to the crucial role of the amount of surfactant in the stability of nanoemulsions formulated according to the low energy method.

This issue was addressed in the current study by the fabrication of a fifth emulsifiable concentrate (Ie) which was made using ingredients from formula (Id) after the reduction in its S/O ratio from 1.0 to 0.5 (Table 1). That means a 50.0% reduction in the amount of surfactant relative to the four other emulsifiable concentrates of set (I). The results showed that this nanoemulsion was physically stable and had particle size < 100 nm even after a 3-month storage period at 20 °C. It is important to note that this result is unattainable in the absence of PG as cosurfactant. This affirms the importance of this compound as a co-surfactant in the formation of physically stable and ethanol-free SEO nanoemulsion, especially at a reduced S/O ratio. On the other hand, at 40 °C, the appearance of this nanoemulsion changed to milky white within 24h, indicating a loss in the nanostructure (Figure 1C). This elicits the temperature sensitivity of (Ie) nanoemulsion as is the case with all nanoemulsions of set (I).

4.3. The effect of HLB on the formation and thermal stability of SEO nanoemulsions

From the above-mentioned results, it can generally be concluded that the common feature among the SEO nanoemulsions of set (I) is their temperature sensitivity under moderate thermal stress (40 °C, Figure 1C**)**. This thermal intolerance is a common phenomenon among nanoemulsions formulated using nonionic surfactants due to their phase inversion which is induced by temperature. The temperature at which the phase inversion of nanoemulsion takes place is called the phase inversion temperature.

At this temperature, the hydrophilic head groups of the nonionic surfactants become dehydrated due to the breaking down of their hydrogen bonds with water. This in return decreases the HLB (hydrophilic lipophilic balance) of the surfactant, making it more hydrophobic. This causes the nanoemulsion to become highly unstable and turn into a cloudy macroemulsion (Kunieda and Shinoda, 1982).

Previous investigations dealt with this issue by incorporating a second surfactant besides the main one in order to modify the HLB to make it appropriate for improving the thermal stability of the nanoemulsion (Guttoff *et al*., 2015). For instance, the previous reference used sodium dodecyl sulfate to act as a second surfactant to improve the thermal stability of vitamin D nanoemulsion. However, that kind of anionic surfactant is not permitted in food and beverage applications.

Therefore, to render SEO nanoemulsions edible, the author of the current study substituted the anionic surfactant with two nonionic food-permitted surfactants which were mixed, separately, with the principle surfactant (Tween 80), to form a food-permitted binary surfactant mixture. Accordingly, two more new sets of SEO emulsifiable concentrates namely, set (II) and set (III), were fabricated as shown in Table (1).

SEO nanoemulsions of set (II) were formulated using a mixture of surfactants composed of Tween 80 and Tween 20 at 3 different weight ratios (Table 1). This surfactant mixture is called family binary because it belongs to the same family of polysorbate surfactants but differs only in the length of the fatty acid moiety. Results showed that the surfactants of set (IIa) with an HLB value of 15.55 gave the nanoemulsion with the smallest particles size (29.5±0.2 nm) compare to IIb and IIc, indicating the suitability of that HLB value for SEO.

Regarding storage stability, all nanoemulsions of set (II) were not subjected to the 3-month storage period at 20 °C as was the case with set (I). This is because set (II) nanoemulsions were designed mainly for investigating the effect of surfactant mixtures and their HLB on the thermal stability of SEO nanoemulsions.

In this regard, none of the 3 nanoemulsions of set (II) was thermally stable at 40 °C, as shown in Figure 2B. This indicates the inability of all ratios of the surfactant mixtures of set (II) to provide the appropriate HLB value necessary for extending the range of phase inversion temperature of the nanoemulsion to provide the required thermal stability (Shinoda and Saito, 1969).

As a result, a second trial was performed to enhance the thermal stability of SEO nanoemulsions by formulating a third set of SEO emulsifiable concentrate (set III) using different a surfactant mixture made of Tween 80 and Cremophor RH40 (Table 1). This mixture is called the hetero binary system because it belongs to different fam-

ilies of nonionic surfactants. The purpose was to achieve an appropriate HLB which could improve the thermal stability of SEO nanoemulsions.

It is important to indicate that the different HLB values which result from mixing the surfactants of set (III) cannot be calculated precisely. That is because the HLB value of Cremophor RH40 is not univocal, which means that the surfactant has a wide range of HLB values ranging from 14-16, as indicated by the manufacturer. This makes it difficult to calculate an exact HLB value for the binary mixture of (Tween 80-Cremophor) at the three different ratios (refer to Table 2).

The results from the fabrication of set (III) showed that nanoemulsions (IIIa) and (IIIb) showed the typical characteristics of nanoemulsion regarding their transparent appearance and ultra-fine particle size (Figure 3A and Table 2). Moreover, these nanoemulsions were more thermally stable and temperature tolerant up to 50 °C compared to all nanoemulsions produced using set (I) & (II), as shownd in the results section. The author justified that thermal stability is based on hitting the right mixture of surfactants (Tween80-Cremophor) in the right ratio (1:1, formula IIIa and 2:1, formula IIIb, Table 1). As a result, an appropriate HLB value was reached which was able to extend the thermal stability of the nanoemulsion (Shinoda and Saito, 1969).

5. CONCLUSIONS

This article investigated in detail the different aspects that can lead to the development of a stable ethanol-free SEO nanoemulsion using the low energy technique. The results reported in this article could have a promising application in developing water-based SEO nanoemulsions for application as flavoring and aromatizing agent in food and beverages. It also draws the attention of relevant researchers to the deferential effect of PG as a cosurfactant in the formation of water-based essential oil nanoemulsions.

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