ACS FOOD SCIENCE & TECHNOLOGY

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High-Pressure-Homogenized Clove and Thyme Oil Emulsions: Formulation, Stability, and Antioxidant Capacity

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Cite This: *ACS Food Sci. Technol.* 2022, 2, 1832−1839

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ABSTRACT: This study focuses on the formulation of clove oil and thyme oil emulsions showing antioxidant capacity. Emulsions were formulated with 2 and 4% of each essential oil by using microfluidization. Tween 80 was used as the surfactant. Droplet sizes and polydispersity index values of emulsions were measured, and their effects on the emulsion stability were investigated. While thyme oil emulsions showed a good stability for 3 months, destability started in clove oil emulsions soon after homogenization due to Ostwald ripening. Stability results were parallel to the change of polydispersity index over time. Clove oil was found to be a more powerful antioxidant than thyme oil. It was also observed that clove oil emulsions had more antioxidant capacity than thyme oil emulsions. Total phenolic content results were in accordance with the antioxidant capacity results. The most significant observation of the study was the increase in droplet size of the clove oil emulsions followed by microfluidization due to the recoalescence phenomena.

KEYWORDS: *clove oil, thyme oil, nanoemulsion, droplet size, antioxidant capacity*

1. INTRODUCTION

Essential oils are the natural compounds derived from different parts of plants, especially from leaves, barks, seeds, flowers, fruits, or roots. They are highly volatile and aromatic compounds. They are widely known to have antioxidant, antibacterial, antifungal, anti-inflammatory, and anticarcinogenic activities.[1] *Syzygium aromaticum*, clove in the common language, is a tree that belongs to the family *Myrtaceae*. Its major component is *eugenol*, although it may change depending on the extraction method and the tree's region. It has been reported that eugenol adds antioxidant, antimicrobial, antiseptic, and anticancer properties to clove oil due to its phenolic structure.[2] *Thymus vulgaris*, thyme in the common language, is a herb that belongs to the Lamiaceae family. Thyme is a naturally growing plant in the Mediterranean region. Although composition of thyme varies depending on the region, thymol and carvacrol are the major constituents.[3] They are phenolic monoterpenes that give antioxidant and antimicrobial properties to the thyme oil.[4]

Nanoemulsions are defined as the emulsion systems which have an average droplet size of 200 nm.[5,6] In terms of stability, emulsions are examined in two ways: *thermodynamic stability and kinetic stability*. Nanoemulsion formation is a thermodynamically unfavorable process. Yet, they are known as kinetically stable systems.[7] Although they have good stability with time, they break down eventually. They mainly break down due to the shape and distribution of droplet sizes, gravity effect, and imbalances between attractive and repulsive forces.[8] Nanoemulsions composed of essential oils mostly suffer from *Ostwald ripening* (OR), which occurs when smaller droplets dissolve and deposit to larger ones and large droplets become larger. OR is usually explained with the solubility difference of droplets due to their radius of curvatures. It has been considered as the major destabilization mechanism in emulsions where the oil phase is relatively soluble in water as in essential oils.[9]

Emulsification techniques can be classified into two groups as high-energy techniques and low-energy techniques. While high-energy methods exert high shear forces, low-energy methods are controlled by the physiochemical interactions between the surfactants and liquids.[10] Emulsification consists of several steps: *disruption of the initially formed droplets, covering of freshly formed interfaces by the emulsifier, collisions, and possible coalescences*. Droplet size reduction occurs due to the balance between droplet break up and recoalescence.[11] Actions of the emulsifier, amount of energy supplied to the system, dispersed phase properties, concentration, viscosity, and temperature are the factors that control the droplet size reduction.[12] More information on the utilization of different emulsification methods has been summarized in recent reviews.[13,14]

Tween 80 is a widely used surfactant in food, cosmetic, and drug industries. It is classified as a nonionic surfactant with an HLB value of 15. Tween 80 is a small molecule that facilitates the adsorption to the interface resulting in a stable emulsion. It is also known as being nontoxic and nonirritating.[15]

Received: August 3, 2022
Revised: October 24, 2022
Accepted: November 2, 2022
Published: November 16, 2022

In this work, clove oil and thyme oil nanoemulsions were produced with the high-energy technique: *microfluidization*. Nanoemulsions were prepared at two different concentrations. Tween 80 was used as the surfactant. The effects of essential oil type, concentration, and microfluidization on the droplet size were investigated. Changes in droplet size during 1 month of storage were recorded. Transmission electron microscopy (TEM) was used for morphological analysis of the nanoemulsions. Antioxidant capacity tests [DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP] and total phenolic content tests were performed on the primary and homogenized nanoemulsions. In this way, the novelty of this study can be described as the comparison of clove oil and thyme oil nanoemulsions. In fact, clove oil and thyme oil nanoemulsions have been studied widely.[16−31] Some of these studies focus on the antimicrobial effect of the clove or thyme oil nanoemulsions. They also differ from this study in terms of surfactant type, emulsification method, and characterization analyses. Moreover, the antioxidant capacity of thyme and clove oils has never been compared except one study in which they were formulated to produce hydrogels.[32] Therefore, this study adds novelty to the clove and thyme oil nanoemulsion studies by making comparison between two types of essential oils.

2. MATERIALS AND METHODS

2.1. Materials. Clove oil and thyme oil were purchased from Botalife (Isparta, Turkey). Tween 80, ethanol, methanol, acetic acid, sodium hydroxide, Folin-Ciocalteu (FC) reagent, and gallic acid were obtained from Merck (Darmstadt, Germany). Trolox, DPPH and 2,4,6 tripyridyl-*S*-triazine (TPTZ) reagents, sodium carbonate, ferric chloride, and sodium acetate trihydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of Emulsions. Emulsions were prepared by mixing essential oil as the dispersed phase and distilled water as the continuous phase. Tween 80 was selected as the surfactant. Emulsions were formulated with two different essential oils (clove oil and thyme oil) at two different concentrations (2% v/v and 4% v/v). The surfactant to oil ratio (S/O) was 1:1.

2.2.1. Preparation of the Primary Emulsions. The essential oil-surfactant-water mixture was prehomogenized with Ultra-Turrax (WiseTis Homogenizer, Witeg Labortechnik GmbH, Germany). This treatment lasted 2 min at 10,000 rpm.

2.2.2. High-Pressure Homogenization (Microfludization) of Emulsions. Prehomogenized emulsions were fed into the microfluidizer (Nano-Disperser, NLM 100, South Korea). The effects of pressure (900 and 1300 bar) and the number of cycles (3 and 5) on stability of emulsions were investigated in the preliminary experiments. The stable samples, which were obtained at a pressure of 1300 bar (130 MPa) with five cycles, were selected for further experiments.

2.3. Characterization of Emulsions. *2.3.1. Droplet Size Analysis.* The droplet sizes of the primary emulsions and high-pressure homogenized emulsions were measured every 2 weeks in 1 month period. All samples were stored in the dark at room temperature. These measurements were conducted with dynamic light scattering (DLS)-based equipment (Malvern Zetasizer, Nano ZS90, Worcestershire, UK). Before each measurement, all samples were diluted to 1:100 ratio with distilled water to prevent possible multiple scattering effects. Additionally, poly dispersity index (PDI) of the results was recorded.

2.3.2. Transmission Electron Microscopy. TEM analysis was conducted for morphological analysis. The images were also used to confirm the droplet size results from DLS. The images were collected using a transmission electron microscope (Tecnai G^2 Spirit Biotwin, FEI Company). A drop of the emulsion was poured into the copper grid. It was let to dry at room temperature. Finally, contrast was created with the electrons accelerated in 120 kV.

2.3.3. Antioxidant Capacity. Antioxidant capacities of primary and high-pressure homogenized emulsions and pure essential oils were determined using DPPH· and FRAP methods.

2.3.3.1. Antioxidant Capacity by DPPH· Method. Antioxidant capacity of the samples was measured spectrophotometrically according to DPPH· method described by Zhang, Guo, Guo, Jiang, and Ji (2018) with some modifications.[33] First, DPPH· solution was prepared by dissolving 5 mg of DPPH· reagent in 200 mL of methanol. 0.1 g of emulsion was dissolved in 1 mL mixture of ethanol/acetic acid/water (50:8:42 v/v). After vigorous shaking, 0.1 mL of each sample was pipetted into aluminum-covered tubes. Finally, 3.9 mL of DPPH· solution was added. Final mixtures were kept for 1 h in the dark. When the incubation time was over, absorbance values were recorded at 517 nm with a UV Spectrophotometer (Optizen Pop Nano Bio, Korea). A calibration curve was plotted using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as the standard. The results were expressed as μmol of Trolox per g of the sample (μmol Trolox/gr sample).

2.3.3.2. Antioxidant Capacity by FRAP Method. Antioxidant capacity of the samples was measured spectrophotometrically according to the FRAP method described by Benzie and Strain (1996) with minor modifications.[34] FRAP solution was freshly prepared each time from the stock solutions: for acetate buffer stock solution, 1.6 g of sodium acetate trihydrate was mixed with 400 mL of water containing 8 mL of glacial acetic acid. The pH of the mixture was adjusted to 3.6 with 1 M NaOH. The mixture is completed to 500 mL with water. TPTZ stock solution was prepared by dissolving 156 mg of TPTZ in 50 mL of 40 mM HCl. For the final stock solution, 50 mL of 20 mM ferric chloride solution was prepared. 2.2 mL of FRAP reagent containing acetate buffer, TPTZ solution, and ferric chloride solution at a ratio of 10:1:1 (v/v/v), respectively, was pipetted into tubes. Then, 20 μL of emulsion was poured to FRAP reagent. The mixtures were kept at room temperature in the dark for 4 min. As the reaction time is reached, absorbance at 593 nm was recorded with a UV spectrophotometer (Optizen Pop Nano Bio, Korea). A calibration curve was plotted using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as the standard. The results were expressed as μmol of Trolox per g of the sample (μmol Trolox/g sample).

2.3.4. Total Phenolic Content. Total phenolic content of the samples was measured spectrophotometrically according to FC method explained by Moisa et al. (2018) with minor modifications.[35] FC reagent was diluted with distilled water (1:100) (v/v). 20 μL of emulsion was poured into 2 mL of the diluted reagent. Following shaking for 3 min, 700 μL of saturated sodium carbonate solution was pipetted into the previous mixture. The final mixture was kept at room temperature for 1 h in the dark. Finally, the color change was detected with a UV spectrophotometer at 760 nm. The same procedure was applied to the gallic acid solutions to obtain the calibration curve. The results were expressed as milligram of *gallic acid equivalent* per gram of the sample (mg GAE/g sample).

2.3.5. Instantaneous and Long-Term Stability Tests. Stability tests were done only for high-pressure homogenized emulsions since primary emulsions are prone to break down easily. They were stored at room temperature.

Instantaneous stability was checked by centrifugation (Micro-Spin12, USA). After pouring 1 mL of samples into mini tubes, the height was recorded. They were subjected to 15,115*g* for 1 min. Height of the supernatant was recorded. The ratio between the initial and final heights was reported as "*instantaneous stability*".[36]

For long-term stability, homogenized emulsions were first observed with the naked eye. Emulsions with any visual separation were not tested further. Long-term stability tests were applied for 3 months.

2.3.6. Viscosity Measurements. Viscosities of pure essential oils were measured with the vibro viscometer (SV10, A&D Company) to understand their behaviors in emulsions. Calibration was done with distilled water at 20 °C.

2.4. Statistical Analysis. All experiments were conducted with three replicates. The results were expressed as mean ± standard error. Statistical analyses were performed by using analysis of variance (ANOVA) with Minitab V19 (Minitab Inc, Coventry, UK). Tukey's

https://doi.org/10.1021/acsfoodscitech.2c00231
ACS Food Sci. Technol. 2022, 2, 1832−1839

test was used as the comparison test at a confidence level of 95%. Pearson correlation analysis was applied for the antioxidant capacity and total phenolic content measurements. Different letters denote significant differences among the samples ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Droplet Size and PDI Values of Emulsions. Droplet size is the most important criteria in emulsion systems since it does not only identify the emulsion type but also give an idea about its stability when measured over time. Droplet sizes of primary and high-pressure homogenized emulsions are given in Figure 1.

This was attributed to the fact that the surfactant and eugenol had similar structures. As seen in Figure 2, while eugenol contains one unsaturated double bond, Tween 80 contains one unsaturated double bond on its oleate chain. The nonpolar chain of Tween 80 created an interaction between similar structures and caused better adsorption of Tween 80 in clove oil emulsions. Better adsorption decreased the interfacial tension at a higher rate and resulted in droplets with smaller sizes for clove oil nanoemulsions. Similar results were obtained in previous studies. Nagaraju et al. (2021) reported that Tween 80 easily adsorbed around the clove oil droplets due to the presence of double bond on its nonpolar chain.[37] Similarly, Edris and Malone (2012) hypothesized that the surfactant-like structure of eugenol increased the surfactant efficiency.[38]

PDI results of the emulsions are given in Table 1. These results indicated that homogenization was sufficient for reducing PDI in all emulsions.

In this study, high-pressure homogenization (*microfluidization*) was applied to form nanoemulsions with small droplets which provide longer stability. However, as seen in Figure 1, microfluidization did not decrease the droplet size except 2% thyme oil emulsions. On the contrary, it caused an increase in droplet size of clove oil emulsions and did not affect the droplet size of 4% thyme oil emulsions. It is known that microfluidization is usually used to obtain emulsions with fine droplets, thanks to its controllable parameters: *pressure and the number of cycles*. By increasing both parameters, it is expected to form much smaller droplets as disruptive forces on the droplets increase.[39] However, increasing pressure or the number of cycles can only decrease the droplet size up to a certain point. In fact, microfluidization might be ineffective and sometimes cause an increase in the droplet size. This phenomenon is usually explained by "*recoalescence*". As stated earlier, there should be balance between droplet break up and recoalescence during the emulsification process.[11] To sustain that balance, the actions of emulsifiers are critical in many ways. If there is not enough emulsifier, not all droplets can be covered by the emulsifier, and they stick with their neighbors. On the contrary, adding too much emulsifier is considered as another problem because electrostatic repulsion weakens and agglomeration starts.[40] Additionally, the emulsifier should adsorb and stabilize the droplets in a very short time. If stabilization is achieved slower than collusions, then recoalescence is observed.[41]

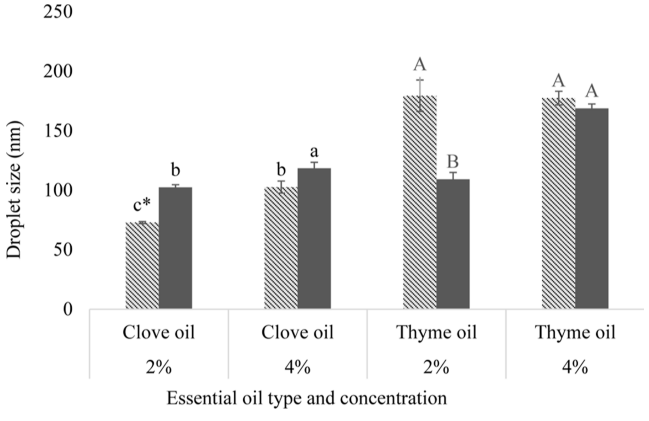

Figure 1. Mean droplet size of primary (▨) and homogenized (■) emulsions at different concentrations. *Different small and capital letters indicate significant differences between different concentrations of clove and thyme oil emulsions, respectively, ($p < 0.05$).

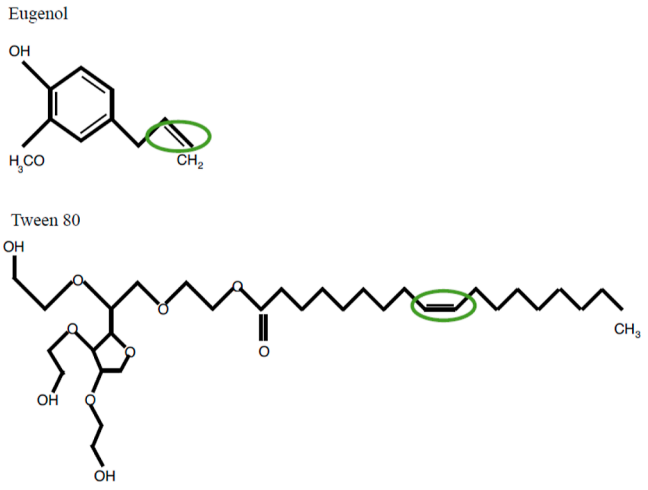

Figure 2. Chemical structures of eugenol and Tween 80. The unsaturated bonds are encircled.

Table 1. PDI Values of Emulsions[a]

sample ID	PDI	sample ID	PDI
C2-P	0.410 ± 0.007[a]	T2-P	0.385 ± 0.002[b]
C2-H	0.212 ± 0.017[b]	T2-H	0.235 ± 0.008[c]
C4-P	0.180 ± 0.004[bc]	T4-P	0.469 ± 0.013[a]
C4-H	0.158 ± 0.013[c]	T4-H	0.128 ± 0.006[d]

[a]PDI values were compared separately for clove and thyme oil emulsions. Different letters indicate significant differences ($p < 0.05$) among the samples in the same column.

Energy input is another critical parameter. There is such a pressure value that the droplet size cannot be reduced further after a point. "Overprocessing" occurs when emulsions are given more than required energy. Over-processed droplets tend to recoalescence and form larger-sized droplets.[10] It is a problem seen in high-energy emulsification techniques, especially in the high-pressure homogenizers. In different studies, different optimum pressures for different emulsion systems were reported in the range of 80−100 MPa.[10,42]

Overprocessing is explained with the coalescence frequency, which itself depends on the factors such as the collision frequency and the collision probability. Coalescence frequency is defined as the number of coalescences per unit volume and unit time. Collision probability can be related to coalescence time which depends on droplet size and energy. Not surprisingly, as the probability of collision and the frequency increases, coalescence increases.[43] Coalescence is highly affected by the viscosity, energy input, and droplet size. The faster the droplets in the channels of high-pressure

https://doi.org/10.1021/acsfoodscitech.2c00231
ACS Food Sci. Technol. 2022, 2, 1832−1839

homogenizers, the more collisions take place. Droplets may reach high velocities when emulsion has low viscosity or when they are given extra energy. Small droplets speed up even more due to higher mobility. Viscosity plays a major role in terms of mobility.[44] At this point, a viscosity comparison between essential oils and vegetable oils can enlighten the problem better. Sunflower oil is one of the most commonly used oil in food emulsions. The viscosity of sunflower oil is measured as 63 cP (at 20 °C).[45] In this study, the viscosity of pure oils was measured at 20 °C as follows: 9.03 and 15.5 cP for clove oil and thyme oil, respectively. When three viscosity values are compared, sunflower oil is much more viscous than both essential oils. In fact, clove oil is the least viscous one by being even quite different than thyme oil. Therefore, one can understand why this recoalescence phenomenon is not common in regular food emulsions. Rather, it is more possible in emulsions with the low-viscous components. It has also been reported in some recent studies that the more viscous emulsions tend to be stable for a longer time.[46,47]

What happened with the clove oil emulsions in this study is an example of "over processing". Due to its low viscosity, droplets of clove oil emulsions accelerated too much in the channels. High mobility of clove oil droplets increased the number of collisions which itself is a parameter of over-processing. Overprocessed clove oil droplets formed larger droplets. It was concluded that droplet size of clove oil emulsions increased after the emulsification process due to excess amount of energy. These results are similar to those reported in a high-pressure homogenization of orange essential oil emulsion.[48]

Microfluidization caused a significant reduction in the droplet size in 2% thyme oil containing emulsion (T2-P) ($p < 0.05$) (Figure 1). Conditions of the homogenization process worked most successfully with 2% thyme oil emulsions. In contrary, no change in the droplet size was observed in 4% thyme oil emulsion. The problem with that sample was thought to be the insufficient amount of the surfactant. Energy dispersion was successful in the 2% thyme emulsion, meaning viscosity was not a problem with thyme oil like it was for clove oil. This indicated droplet size first decreased; however, the surfactant could not cover all droplets, and it coalesced with the surrounding droplet. Consequently, droplet size remained unchanged.

3.2. TEM Results. TEM is a widely used method to confirm the droplet size results. According to Figure 3, it was proven that emulsion droplets were spherical in shape with a desired nanometric diameter size. Additionally, transmission electron micrographs of primary and homogenized emulsions were almost in accordance with the droplet size results. There is a slight difference between DLS results and TEM results as TEM gave a slightly smaller value. This is expected because it is known that drying of samples for TEM analysis may cause shrinkage.[49]

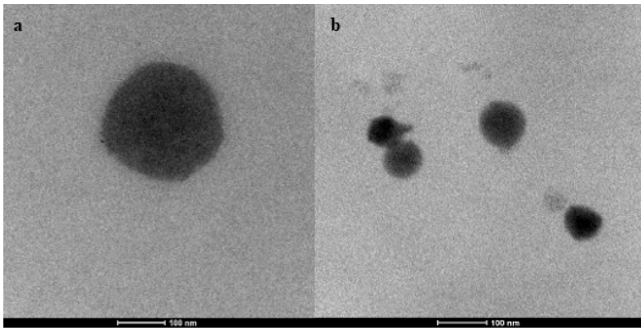

Figure 3. Transmission electron micrographs of primary (a) and homogenized (b) 2% thyme oil emulsions with a scale of 100 nm.

3.3. Antioxidant Capacity. *3.3.1. Antioxidant Capacity of Pure Essential Oils by DPPH and FRAP Methods.* In the DPPH method, while pure clove oil showed 1213.8 ± 5.2 μmol Trolox/g oil, thyme oil showed 11.3 ± 0.1 μmol Trolox/g oil antioxidant capacity. In the FRAP method, while pure clove oil showed 1734.5 ± 110.7 μmol Trolox/g oil, thyme oil showed 13.9 ± 0.2 μmol Trolox/g oil antioxidant capacity. The results of both methods were consistent with each other. Clove oil has been identified as the most powerful antioxidant in many comparative studies conducted with BHT, BHA, and Trolox.[2,50] Moreover, it has been reported many times in previous studies that clove oil has much stronger antioxidant property than thyme oil.[51−53]

3.3.2. Antioxidant Capacity of Emulsions. Change in antioxidant capacity between the primary and homogenized emulsion samples after exposing to high pressure (~130 MPa) is given in Table 2.

According to the DPPH· method results, microfluidization did not affect emulsions' antioxidant capacity ($p > 0.05$). Primary and homogenized emulsions showed the same antioxidant capacity both in clove and thyme oil emulsions. The result is important because after such mechanical exposure, essential oils maintained their antioxidant capacity. The FRAP method gave similar results only with one exception. Microfluidization appears to cause a decrease only in 4% clove oil containing the primary emulsion (C4-P) sample. Other emulsions seem unaffected ($p > 0.05$). The difference between DPPH· and FRAP methods arises from the fact that they have different mechanisms. Free radicals are neutralized either by electron or hydrogen atom transfer in the DPPH· method. On the other hand, FRAP is an assay that measures reducing ability of antioxidants where they donate an electron and reduce Fe^{3+} to Fe^{2+}. Chemicals used and the environment are also quite different from each other.[54] During the experiments, essential oils were put into emulsions at two different concentrations (2 and 4%). According to the results, as concentration increased, antioxidant capacity increased in clove oil emulsions in both methods ($p < 0.05$). The change was not an unexpected result as clove oil is known to have high

Table 2. Antioxidant Capacity Assessed by DPPH· and FRAP Assays and Total Phenolic Content of Clove and Thyme Oil Emulsions[a]

sample ID	DPPH·(μmol Trolox/g emulsion)	FRAP (μmol Trolox/g emulsion)	FC (mg GA/g emulsion)
C2-P	12.02 ± 0.08^{b}	42.58 ± 4.02^{c}	6.79 ± 0.50^{c}
C2-H	11.98 ± 0.06^{b}	39.60 ± 3.39^{c}	6.12 ± 0.29^{c}
C4-P	120.51 ± 0.52^{a}	116.72 ± 7.64^{a}	19.36 ± 1.90^{a}
C4-H	118.71 ± 1.56^{a}	85.99 ± 7.35^{b}	15.26 ± 0.75^{b}
T2-P	0.84 ± 0.02^{a}	0.22 ± 0.02^{b}	3.20 ± 0.20^{b}
T2-H	0.84 ± 0.02^{a}	0.22 ± 0.01^{b}	2.64 ± 0.13^{c}
T4-P	0.90 ± 0.04^{a}	0.33 ± 0.03^{a}	6.28 ± 0.47^{a}
T4-H	0.88 ± 0.04^{a}	0.35 ± 0.03^{a}	3.02 ± 0.16^{bc}

[a]Different letters indicate significant differences ($p < 0.05$) among the samples within each experiment. Analysis was conducted separately for different oils.

https://doi.org/10.1021/acsfoodscitech.2c00231
ACS Food Sci. Technol. 2022, 2, 1832−1839

antioxidant capacity which means even changes in small amounts could change the capacity.[55,56] However, when DPPH· results were examined, there was no significant change for thyme oil emulsions ($p > 0.05$). On the other hand, according to FRAP results, thyme emulsions exhibited a higher antioxidant capacity when concentration increased ($p < 0.05$). Overall, when the correlation was checked, there was a strong positive correlation ($r = 0.950$) between DPPH· and FRAP methods for clove oil emulsions ($p < 0.05$). However, there was no significant correlation between DPPH· and FRAP methods for thyme emulsions. These values of correlations explain the differences between the results of two assays which can be summarized by the hydrogen transfer (DPPH·) or single-electron transfer (FRAP). Moreover, limitations of the FRAP method should be considered in the case of thyme oil. It has been reported that the FRAP method is limited to water-soluble compounds and nonpolar compounds may not interfere with the FRAP reagent.[57,58] As it will be discussed in the coming parts, thyme oil is known to have very low water solubility.[59] The high hydrophobic property of thyme oil could cause slow kinetics in the FRAP-reducing reactions. The standard time of the reaction (4 min) might not be enough for the complete titration.

When antioxidant capacities of pure essential oils and nanoemulsions are compared, it was seen that nanoemulsions showed lower antioxidant capacity. This is attributed to the small amount of essential oils in the formulation of nanoemulsions.

3.4. Total Phenolic Content with FC Method. *3.4.1. Total Phenolic Content of Pure Essential Oils.* Phenolic content measurement is critical for antioxidants because this property is commonly associated with the phenolic structure.[35] The total phenolic contents of pure clove oil and thyme oil were 409.9 ± 26.8 mg GA/g oil and 167.2 ± 2.8 mg GA/g, respectively. These results prove that clove oil has more phenolic content and it shows more antioxidant capacity than thyme oil ($p < 0.05$). It also explains the clove oil being a stronger antioxidant because the antioxidant property is directly related to phenolic content. Turgay and Esen (2015) found out that clove oil showed the highest antioxidant capacity together with the highest total phenolic content among six different plants including thyme, clove, and ginger.[60]

3.4.2. Total Phenolic Content of Emulsions. As seen in Table 2, the phenolic content of thyme oil emulsion seemed to be affected by microfluidization. For clove oil emulsions, on the other hand, only the one with 4% clove oil was affected.

As concentration increased, the total phenolic content increased in all samples as expected ($p < 0.05$).

3.4.3. Correlation between FC Method and Antioxidant Capacity Methods. Phenolic content measurement is critical for antioxidants because this property is commonly associated with the phenolic structure.[35] Therefore, *Pearson correlation* values were checked between the antioxidant capacity results and FC results. Overall correlation results showed that there was a strong positive correlation between the FC and FRAP results for clove oil emulsions (0.977). The correlation between FC and DPPH· was also similar (0.963). Although thyme oil correlations were not high, there was still a positive correlation between FC and antioxidant capacity measured with the FRAP method (0.486). However, no significant correlation was found between FC and DPPH· results for thyme oil.

The good correlation for clove oil is important because it justifies the fact that phenolic structure provides the antioxidant property. On the other hand, this was not the case for thyme oil. Because clove oil is mostly composed of phenolics (eugenol), thyme oil also contains nonphenolic structures such as γ-Terpinene, *p*-Cymene, or 1,8-Cineole that show antioxidant activity.[61] Several studies have shown the antioxidant activity of these compounds[62−64]

3.5. Stability. Once homogenized emulsions were formed, they were centrifuged immediately to see the instantaneous stability. All samples gave an average value of 98−100% height ratios indicating high stability. Long-term stability was checked with droplet size measurement and visual analysis.

The droplet size of the emulsions was measured every 2 weeks during 1 month period. According to the results given in Table 3, while the droplet size of clove oil emulsions increased within 15 days, thyme oil seemed to be stable during 30 days of storage. Based on the visual appearance given in Figure 4, homogenized clove oil emulsions start to destabilize after 1 month. There was a distinct phase separation after 3 months. Since clove oil is denser than water, clove oil moved to the bottom. The appearance of homogenized thyme oil emulsions did not change during 3 months.

As given in Table 4, PDI values of emulsions were also recorded each time the droplet size was measured. PDI has been mostly reported to be related with the stability of the emulsion systems.[37] According to the results, there was no change in PDI values for thyme oil emulsions. On the contrary, there was an increase in PDI values of clove oil. PDI changing within 15 days indicated the start of destabilization.

Emulsions break down with several mechanisms. They are more resistant against gravitational separations because *Brownian motion* dominates the gravitational forces. OR is inevitable in emulsions containing partially soluble oils such as short-chain triglycerides or essential oils.[65] In such emulsions, small droplets have greater solubility than larger ones because when curvature increases, solubility increases. With time, smaller droplets dissolve, and their molecules deposit on the larger molecules.[66,67] In that regard, it can be minimized by obtaining monodisperse emulsions. However, while curvatures exist, OR is highly possible to occur. Considering that clove oil emulsion was unstable, it can be concluded that OR occurred, resulting in coalescence at the end. Authors compared the solubility of thymol, carvacrol, and eugenol and found that

Table 3. Change in the Droplet Size of Clove Emulsions within 1 Month Storage at Room Temperature[a]

	droplet size (nm)		
sample ID	1st day	15th day	30th day
C2-P	72.85 ± 0.84^{c,B}	189.03 ± 15.66^{a,A}	180.57 ± 1.21^{a,A}
C2-H	102.33 ± 2.32^{b,B}	175.50 ± 10.67^{a,A}	186.77 ± 11.53^{a,A}
C4-P	102.55 ± 5.04^{b,B}	183.50 ± 3.88^{a,A}	175.37 ± 2.32^{a,A}
C4-H	118.40 ± 5.06^{a,B}	183.87 ± 4.08^{a,A}	174.00 ± 7.01^{a,A}
T2-P	179.37 ± 13.19^{a,A}	176.67 ± 12.38^{a,A}	164.40 ± 11.5^{a,A}
T2-H	109.17 ± 5.76^{b,A}	115.23 ± 5.54^{b,A}	110.80 ± 3.92^{b,A}
T4-P	177.35 ± 5.74^{a,A}	172.10 ± 5.11^{a,A}	181.60 ± 5.39^{a,A}
T4-H	168.70 ± 3.72^{a,A}	170.10 ± 2.26^{a,A}	175.53 ± 1.05^{a,A}

[a]Different small letters indicate significant differences ($p < 0.05$) among the samples in the same column; capital letters indicate significant differences ($p < 0.05$) among the samples in the same row. ANOVA was conducted separately for different oils.

https://doi.org/10.1021/acsfoodscitech.2c00231
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Figure 4. Photos of emulsions after 3 month storage at room temperature.

Table 4. PDI Values of Homogenized Emulsion Oil Stored over 1 Month at Room Temperature

sample ID	1st day	15th day	30th day
C2-H	0.212 ± 0.017[ba]	0.280 ± 0.024[a]	0.317 ± 0.012[a]
C4-H	0.158 ± 0.013[b]	0.315 ± 0.021[a]	0.285 ± 0.011[a]
T2-H	0.235 ± 0.008[a]	0.260 ± 0.001[a]	0.235 ± 0.001[a]
T4-H	0.128 ± 0.006[a]	0.114 ± 0.010[a]	0.112 ± 0.002[a]

[a]Different letters indicate significant differences ($p < 0.05$) in the same row; all emulsion types were analyzed individually.

eugenol had the highest solubility in water at room temperature.[68] Another study demonstrated that eugenol had higher solubility in water than carvacrol.[69] These results are important in terms of thymol and carvacrol and eugenol being the major constituents of thyme and clove essential oils, respectively. To conclude, during the storage, especially smaller droplets of clove oil emulsion were dissolved, and repulsion was not strong enough to avoid OR and coalescence. Besides, there are many studies which observed instability in clove oil emulsions.[47,70]

4. CONCLUSIONS

High-pressure homogenization is usually applied to decrease the droplet size of the emulsions. However, in this study, it was shown that this high-energy application may not result in a droplet size decrease for all dispersed phases. Smaller droplets were obtained with thyme oil following homogenization. However, an increase in droplet size was observed after microfluidization in clove oil emulsions. It was hypothesized that recoalescence was observed in the homogenization chamber due to the "high energy". Therefore, droplets accelerated unnecessarily and became larger by colliding with each other.

Long-term stability was critical for this study as essential oils are very volatile compounds. It was concluded that changes in PDI values could be an indicator of instability. While PDI values of clove oil emulsions were changing, they were constant for thyme oil emulsions. Consequently, thyme oil emulsions were stable for longer time. However, destabilization started in clove oil emulsions right after homogenization. High solubility of clove oil in water caused OR.

Moreover, antioxidant capacity tests were both applied to emulsions and pure essential oils. Overall, emulsions still had remarkable antioxidant capacity after homogenization. Clove oil emulsions showed higher antioxidant capacity. Good correlation was detected between the phenolic content and antioxidant capacity of clove oil. In the case of thyme oil, a relatively lower correlation was found. This study suggested that essential oils are very good alternatives as natural antioxidant agents. However, their usage is limited with the dosage. Therefore, emulsification is a logical method for essential oils to be used in lower concentrations. However, emulsions should be formed in a way that they show better stability. In that regard, surfactant type and solubility of essential oil should be considered. Therefore, as the outcome of this study, it can be concluded that high-pressure homogenization (microfluidization) is not always the best choice to obtain a stable emulsion with essential oils and using Tween 80 as the surfactant.

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Notes

The authors declare no competing financial interest.

Novelty Impact Statement: this paper adds novelty to the clove and thyme oil nanoemulsion literature by comparing the physiochemical aspects of the nanoemulsions of the two types of essential oil from a different perspective. This study is also novel in that it points out to droplet recoalescence phenomenon during emulsification.

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