FORMULATION AND CHARACTERIZATION OF CLOVE AND THYME OIL EMULSIONS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

FEBRUARY 2021

Approval of the thesis:

FORMULATION AND CHARACTERIZATION OF CLOVE AND THYME OIL EMULSIONS

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ABSTRACT

FORMULATION AND CHARACTERIZATION OF CLOVE AND THYME OIL EMULSIONS

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February 2021, 105 pages

Essential oils are natural aromatic compounds obtained from various parts of plants such as flowers, bark, stems, and roots. They have been used in medicine, cosmetics, and food science since ancient times due to their antimicrobial, antioxidant, and therapeutic properties. Especially in recent times, negative impressions about synthetic additives have increased the use of essential oils in the food industry. The aim of this study is to produce and characterize emulsions with thyme and clove essential oils as natural additives. Emulsification was important to have stable systems since essential oils are highly volatile and aromatic droplets. Through the emulsions, excessive consumption of essential oils was avoided, and long-term stable emulsion systems were obtained while aiming to have same effects. Emulsions were prepared by using essential oil as the dispersed phase and water-surfactant mixture as the continuous phase. As the surfactant, Tween 80 was used. In total, four emulsions were formulated with 2% and 4% of each essential oil by using microfluidization as the homogenization method. Droplet sizes, polydispersity index and zeta potential values of emulsions were measured, and

their effects on stability were investigated. The morphology of the emulsions was analyzed by TEM. Antioxidant capacity and total phenolic content were determined on both pure essential oils and emulsions. In addition, TD-NMR analysis was conducted to measure the relaxation times of T_1 and T_2 of both pure oils and emulsions. As a result of droplet size measurements, emulsions with smaller droplet size were obtained with clove oil emulsions. The reason was thought to be the good interaction between clove oil and Tween 80. However, while thyme oil emulsions were stable for three months, de-stability started in clove oil emulsions soon after homogenization. The mechanism of instability was thought to be Ostwald ripening, which is quite common in emulsions containing essential oils. Stability results were parallel to the change of polydispersity index over time. However, no correlation was found between zeta potential and stability. Considering antioxidant experiments, clove oil was found to be a more powerful antioxidant than thyme oil. It has also been stated that clove oil emulsions have more antioxidant capacity than thyme oil emulsions. The total amount of phenolic content gave a positive correlation with the antioxidant capacity results. In particular, a stronger correlation was found with clove oil.

Keywords: Emulsions, Essential Oils, Droplet Size, Antioxidant Capacity, Stability

ÖZ

KARANFİL VE KEKİK YAĞLARI İÇEREN EMÜLSİYONLARIN FORMÜLASYONU VE KARAKTERİZASYONU

Tonyalı Karslı, Gökcem
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Şubat 2021, 105 sayfa

Esansiyel yağlar bitkilerin çiçek, kabuk, sap ve kök gibi çeşitli yerlerinden elde edilen doğal aromatik bileşiklerdir. Gösterdikleri antimikrobiyal, antioksidan ve tedavi edici özellikleri sebebiyle eski çağlardan beri tıp, kozmetik ve gıda alanlarında kullanılmışlardır. Özellikle son zamanlarda sentetik katkı maddelerine karşı yerleşen olumsuz düşünceler esansiyel yağların gıda endüstrisinde kullanımını oldukça arttırmıştır. Bu çalışmanın amacı kekik ve karanfil esansiyel yağlarıyla oluşturulmuş emülsiyonların gıda ürünlerinde doğal katkı maddesi olarak yer almasını sağlamaktır. Yağlar oldukça uçucu ve kokulu damlacıklar olduğundan çalışmada homojenize emülsiyonlar üretilmiştir. Böylelikle, esansiyel yağların fazla tüketimi azaltılmış ve uzun süre stabil yağ sistemleri elde edilmiştir. Emülsiyonlarda iç faz olarak esansiyel yağlar, dış faz olarak sürfektan ve saf su kullanılmıştır. Sürfektan olarak Tween 80 kullanılmıştır. Hacimce %2 ve %4 yağ içeren toplam 4 farklı formülasyona sahip emülsiyonlar mikroakışkanlaştırma yöntemiyle yüksek basınç altında homojenize edilmiştir. Emülsiyonların, parçacık boyutları, polidispersite indeksleri ve zeta potansiyel değerleri ölçülmüş, bunların

stabilite üzerine etkilerine incelenmiştir. Emülsiyonların morfolojisi TEM ile analiz edilmiştir. Çokca antioksidan özellik gösterdiği bilinen bu yağlara ve emülsiyonlara antioksidan ve toplam fenolik madde testleri uygulanmış ve aralarında bir korelasyon olup olmadığına bakılmıştır. Ayrıca, Zamansal Alanda NMR Relaksometre kullanılarak T1 ve T2 relaksasyon zamanları ölçülmüştür. Karanfil yağı emülsiyonları ile daha küçük çaplı parçacıklara sahip emülsiyonlar elde edilmiştir. Sebebi karanfil ve sürfaktanın daha uyumlu olması olarak düşünülmüştür. Ancak kekik yağı emülsiyonları 3 ay boyunca dağılmadan durabilmişken, karanfil yağlı emülsiyonlarda homojenize edildikten kısa süre sonra bozulmalar başlamıştır. İnstabilite mekanizmasının esansiyal yağ içeren emülsiyonlarda oldukça yaygın olan Ostwald olgunlaşması olduğu düşünülmüştür. Stabilite sonuçları, polidispersite indekslerinin zamanla değişimi ile paralellik göstermiştir. Ancak zeta potansiyel değerlerinin stabilite ile bağına rastlanmamıştır. Yapılan antioksidan deneyleri sonucu karanfil yağının kekik yağından daha güçlü antioksidan ajanı olduğu görülmüştür. Ayrıca karanfil yağı emülsiyonlarının da kekik yağı emülsiyonlarına göre daha çok antioksidan maddeye sahip olduğu belirtilmiştir. Toplam fenolik madde miktarı antioksidan aktivite sonuçlarıyla pozitif korelasyon vermiştir. Özellikle karanfil yağında daha güçlü bir korelasyon tespit edilmiştir.

Anahtar Kelimeler: Emülsiyonlar, Esansiyel Yağlar, Parçacık Boyutu, Antioksidan Aktivite, Stabilite

Without thinking for a second, to my family…

ACKNOWLEDGMENTS

I owe this entire thesis to my advisor, Assoc. Prof. Dr. Mecit Halil Öztop. He knows how much I respect and admire him. Here, I should write it once more so that everybody knows how amazing person he is. Working with him is a prerogative but working with him with lots of fun is another thing. I believe that so far, I have been experiencing the second one. That doesn't mean this whole master period was comfortable. On the contrary, I went through really harsh times in both my academic and personal life. But I can honestly tell that he was there for me in all my breakdowns. He was there not only as an advisor but also as a friend. There were times that I did not believe in myself, but he did as being both. That is all that I want to say rather than the fact that he is the reason why I chose the academic way. The latter is already a known fact because even I was a first-year student, I was following him. Since then, he has supported me and helped me countless times. So, if I try to express my respect to him here, it would be a very long thesis. Therefore, I have to stop here with my sincere thanks. Also, I want to thank my co-advisor, Prof. Dr. Serpil Şahin. I always felt her trust in me. I am grateful for her patience and support during my study.

These are literally the last sentences that I am writing for this thesis. Also, the sentences I have difficulties writing at most. Because whatever I write here will be insufficient to explain the friendship between us. Elif is the best thing that METU brought me. She was with me during my whole thesis period. I asked her for help countless times, and she didn’t turn me down even once. This is not an incredible thing because I know helping each other is a regular consequence of our friendship. I, of course, thank her for the help. But what I am especially grateful for is this friendship that I may not be grateful for her help.

My Bağlıca fellows: I would be rich now if I put a coin aside every time Serap Namlı told me ‘relax, this is thesis, you will finish it like we all did’. Sometimes I listened to her, sometimes not. But, here we are. You were right my friend. Thank you for believing in me. Şahin Namlı is my study-stress soul mate. He understands me, I understand him. By the way, I should admit that sometimes he is funnier than me but only sometimes. I especially thank them for waiting for me so long to play our favorite game Catan. I know this is so valuable. I always told my friends that Seray Kahraman was one of the few people who wrote to me when I was in Erasmus. Since then, I know that I am a special person for her, and she is special for me. I like her being so naive and goog-intended friend. I miss our coffee talks.

Knowing Derya Ucbaş is one of the most important things my master’s life brought me. I can literally say she is one of the reasons why I wanted to work in our department. I love and chose my subject after we worked together on a project with a similar research area. She taught me everything she knew like no one else would do. This is nothing compared to her real friendship. I always feel that.

My pop culture friend, Emrah Kırtıl. With his leaving, I am losing Mr. I hope him to be at the department so that we could enjoy the day. I admire his both academic and Britney knowledge. Finally, I have to say that he always smelt like a perfume and emulsion lotion in our little circus.

My book friend, Eda Yıldız. Also, my quarantine friend. I can name our crazy antioxidant activity days as ‘Experiments in Corona Days’. I especially want to thank her for keeping our teapot ready. That means a lot to me.

My master soldiers: Eylül Uygun, Gökhan Uğur Atıl, Öznur Sezgin and Mohammad Bin Zia. We were together in this fight. We were having so much fun during the courses we took together. I already miss Gökhan’s shows. I miss us criticizing video clips with Eylül. I miss my ‘lumpen friend Gülben Ergen’. I cannot tell how nice things MBZ did for me. He is my travel buddy. I miss his presence in Ankara.

My special thank is for Selen Güner. She is the one who taught me the vital equipment I used because we had similar study areas. Additionally, she was the one I immediately called whenever I had a problem with the equipment. She answered me every time and solved my problem.

My other siblings: Nehir Alaz and Ali Alaz. We grew up together. Nehir is now 15 but she has always been more mature than me. She always calms me with her wisdom. Since she was a baby, there is a connection between us that I cannot explain. During my master's education, there were at least three times when I felt horrible and got a surprise present from her. Somehow, she was healing me with this punctual instinct. Ali is like my brother, who always makes me cry with his sensuality that he tried not to reflect. Everybody knows that I love them so much.

How can I express my gratitude towards my family? It is impossible. I have the best parents in the world. They did nothing but sacrifice for us. I know how proud my father is just because we both graduated from the same university with him, at the expense of staying away from us. I know how many nights my mother couldn't sleep because she was thinking about us. I know only they always wanted what's good for us. I know I am nothing without them. My sister, Bade. Actually, she is my third parent because she was always making sacrifices by becoming more than a sister. She is so devoted that she has always given me priority since my childhood. I could become the 'spoiled small child' thanks to her. Even better, I still don't have to grow up because she still makes me feel like one. Having a sister who cares about me more than I do myself is beyond everything. Finally, I have to admit that her contribution to this thesis as a food engineer is invaluable.

And my dear love, Hakan: The family I chose. He is the best chance of my life. He is my wisdom guy. I learned to be patient and make sacrifices, thanks to him. I cannot say he helped me. What he did cannot be identified as to help. Because his 'help' to me would make him get a degree from the food engineering department. I am grateful to my husband!

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CHAPTER 1

INTRODUCTION

1.1 Essential Oils

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, as the name indicates. The aroma and odor of the essential oils depend on the constituents present in the plant (Tongnuanchan & Benjakul, 2014). These constituents form the major compounds that are classified as terpenes and oxygenated compounds. Terpenes are found mostly in the form of monoterpenes such as limonene and pinene. Oxygenated compounds can be considered under the group of terpenoids, which are also derived from terpenes. Thymol, carvacrol, and eugenol are some examples for this group. They all have phenolic structures (Nazzaro, 2017; Tongnuanchan & Benjakul, 2014). Some common terpene structures are given in Figure 1.1.

Even in the early times, the use of essential oils was frequent due to their functional properties. They are widely known to have antioxidant, antibacterial, antifungal, anti-inflammatory, anti-carcinogenic activities, and so on (Nazzaro, 2017). Depending on the concentrations of biological compounds they include, each of them may show different properties.

There are various extraction methods for essential oils: cold expression, solvent extraction, steam distillation, or water distillation. However, steam distillation is the most commonly used method for conventional purposes (Stratakos & Koidis, 2016).

A wide variety of essential oils exhibit antimicrobial activity. Their activity may change depending on the composition and functional groups they include. They

show different effects on different strains of microorganisms. The mode of action is based on their potential to disrupt the cell walls and then, cell membrane. The permeability increases, resulting in leakages of the compounds such as some critical ions and proteins (Akthar, Degaga, & Azam, 2014). Once they are in the cell, they can disturb the mitochondria and inhibit ATP generation. Eventually, they may lead cells to lysis and cause cell death (Nazzaro, 2017).

In general, cell walls of microorganisms become the main target because their fat-based outer layers match with the lipophilic structure of essential oils (Anna K Jager, 2014). In that regard, Gram-positive bacteria are known to be more susceptible to essential oils than Gram-negative bacteria. Because Gram-positive bacteria cell walls consist of thick layers of peptidoglycan, which makes them lipophilic (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). Therefore, essential oils can easily penetrate through the cell wall and go on acting in the cytoplasm. However, Gram-negative bacteria cell walls consist of a thinner layer of peptidoglycan surrounded by an outer membrane. The outer membrane is almost impermeable to hydrophobic molecules (Chouhan, Sharma, & Guleria, 2017). This clarifies the different resistances of bacteria show.

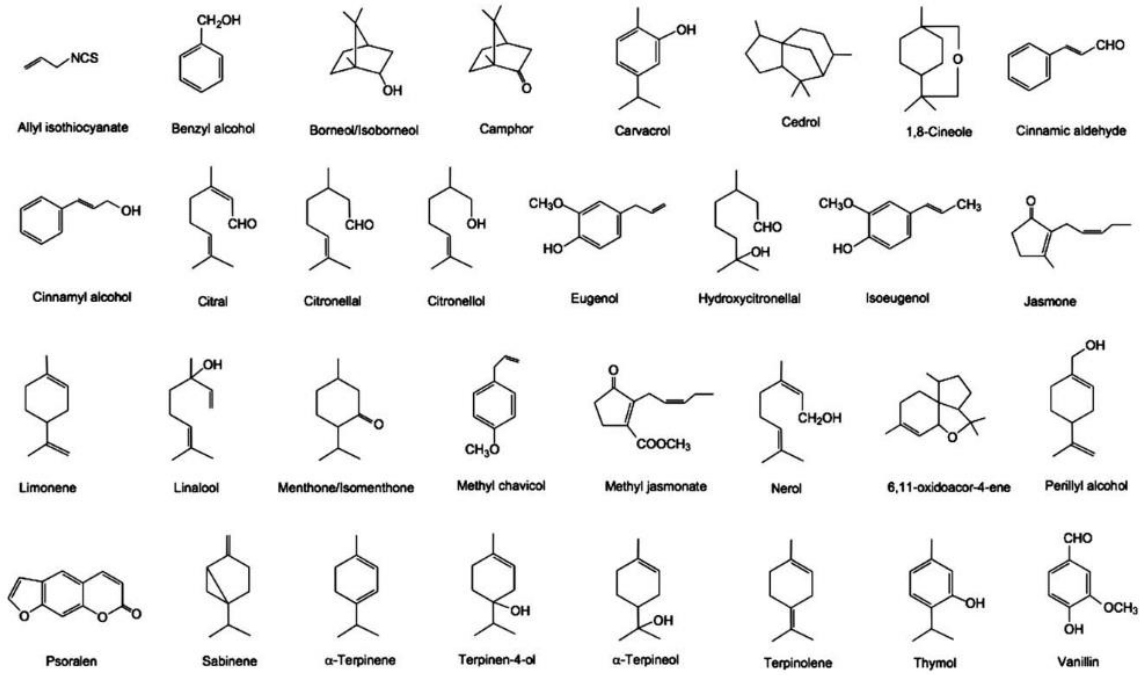


Figure 1.1: Some examples for terpene structures (Shaaban, El-Ghorab, & Shibamoto, 2012)

1.1.1 Thyme Oil

Thymus vulgaris, thyme in the common language, is a herb that belongs to the *Lamiaceae* family, which contains approximately 400 other species. Thyme is a naturally growing plant in the Mediterranean region (Borugă et al., 2014). A typical thyme plant can be seen in Figure 1.2. Its leaves are frequently used as a culinary herb all over the world. Apart from its use in the kitchen, both itself and its oil as extract have several applications in different areas. In some countries, they play a vital role in traditional medicine. Since ancient times, it has been used to cure respiratory diseases (Fachini-Queiroz et al., 2012). Besides, they are widely used in pharmacy, cosmetic, and food industries as calming, preservative, and aromatic agents (Salehi et al., 2018).

Figure 1.2: Thyme plant

Thyme oil is most commonly extracted with steam distillation. Although its composition varies depending on the region that thyme grows, thymol and carvacrol are the major constituents generally. Their structures are given in Figure 1.3. They are phenolic monoterpenes that give such bioactivities to the thyme oil (Komaki, Hoseini, Shahidi, & Baharlouei, 2016). It has been verified in numerous studies that antioxidant and antimicrobial properties of thyme oil come from these components (Amiri, 2012; Nickavar, Mojab, & Dolat-Abadi, 2005).

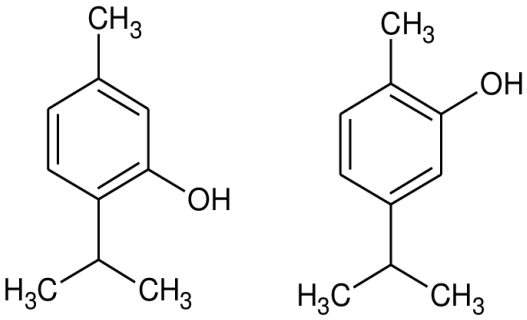


Figure 1.3: Structures of thymol and carvacrol

1.1.2 Clove Oil

Syzygium aromaticum, clove in the common language, is a tree that belongs to the family Myrtaceae naturally found in Indonesia. It is an evergreen tree that can grow up to 2-10 meters long (J. Singh, Baghotia, & Goel, 2012). The part we know as clove is actually the flower buds of this tree which is given in Figure 1.4. Since ancient times, it has been used to remedy oral diseases and dental complaints (Cai & Wu, 1996; Wankhede, 2011). Apart from these, many other applications exist. Traditionally, it is also used in the treatment of vomiting, nausea, and stomach disorders. Its usage as a fragrance by the cosmetic and detergent industry is quite common worldwide (Mbaveng & Kuete, 2017). In the food industry, it is used as flavoring and preservative agents.

Figure 1.4: Clove plant

Clove oil is obtained from the dry flower bud. Its major component is eugenol, which constitutes almost 80% although it may change depending on the extraction method and the tree's region (Gulcin, 2011). It has been reported so often that eugenol adds antioxidant, antimicrobial, antiseptic, and anticancer properties to clove oil (Mohammadi Nejad, Özgüneş, & Başaran, 2017). Structure of eugenol is given in Figure 1.5.

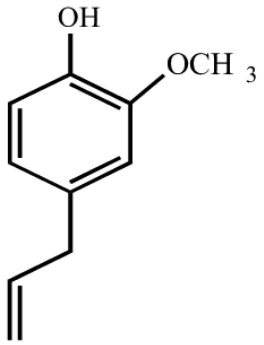


Figure 1.5: Structure of eugenol

1.2 Antioxidants

Antioxidants are agents with the ability to delay or prevent oxidation and reduce oxidative stress. Oxidative stress is the imbalance state between free radicals and antioxidants. Free radicals are highly unstable molecules with an odd number of electrons. They are examined in two groups as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Charles, 2013). Free radicals are formed with both exogenous and endogenous activities. While consumption of cigarettes and alcohol, pollution, heavy metals, or radiation are examples of exogenous reasons, aging, inflammation, cancer, or stress are examples of endogenous reasons (Pizzino et al., 2017). Normally, they should be in balance within the cells. However, when the balance shifts away towards free radicals, they accumulate and cause some disorders. It has been reported so often that excessive oxidative stress is involved in the reasons for cancer, Alzheimer's, aging, diabetes, heart diseases, etc. (Charles, 2013; Gülçin, 2012; Prior, Wu, & Schaich, 2005).

Antioxidants prevent or slow down the oxidation by being oxidized themselves. Their action of mode is based on either breaking the chain reactions or preventing the reactions (Atta, Mohamed, & Abdelgawad, 2017). Chain breaker antioxidants interact with free radicals and transfer an electron or hydrogen atom to free radical.

After impairing, free radicals become stable molecules. Since stable molecules do not undergo further reactions, the oxidation process is terminated (Jain & Sharma M. P., 2011). Preventative antioxidants primarily chelate metal ions such as copper or iron as well as scavenge free radicals. By so, oxidation and possible tissue damage are prevented (Gülçin, 2012; Mehta & Gowder, 2015). The most commonly used antioxidants are BHT (butylated hydroxytoluene), vitamin E, vitamin C, α-tocopherol, carotenoids, and flavonoids.

Natural and synthetic compounds with phenolic groups show antioxidant properties. These compounds are known for their good hydrogen donating ability (Lobo, Patil, Phatak, & Chandra, 2010). Antioxidant properties of essential oils arise from their phenolic content. For example, thyme oil and clove oil mainly consist of thymol and eugenol, respectively. Thymol and eugenol have phenolic rings in their structures (Amorati, Foti, & Valgimigli, 2013). Essential oils can serve as chain breakers in the oxidation steps (Amorati et al., 2013). They have high reactivity towards peroxyl radicals. In the propagation step, they donate an H atom from the phenolic hydroxyl group to peroxyl radicals ($ROO^{\cdot}$) (Baschieri, Ajvazi, Tonfack, Valgimigli, & Amorati, 2017)

1.2.1 Methods for Determination of Antioxidant Capacity

1.2.1.1 DPPH Radical Scavenging Method

DPPH (2,2-diphenyl-1-picrylhydrazyl radical) is a stable free radical reagent with a violet purple color. It shows high absorption at 517 nm (Akar, Küçük, & Doğan, 2017). The reaction between the antioxidants and DPPH radical causes a color change from violet purple to yellow. The color change is observed with UV-Visible Spectrophotometer. More color change indicates higher antioxidant activity. The reaction mechanism between the DPPH radical and antioxidant may differ. The reaction occurs either with the abstraction of a hydrogen atom or electron transfer

from the antioxidant (Kedare & Singh, 2011; Miguel, 2010). Therefore, DPPH becomes stable, as its electron pairs off (Kedare & Singh, 2011).

DPPH method is one of the most popular methods among antioxidant assays because it is an inexpensive and quick method. Reaction occurs slowly, allowing DPPH to react with all antioxidant compounds in the sample, even the weak ones (Kedare & Singh, 2011).

However, it has some limitations. DPPH is a very sensitive reagent. It is easily affected by the light, oxygen and changes in pH (Ozcelik, Lee, & Min, 2014). Therefore, experiment should be conducted in a controlled environment.

1.2.1.2 FRAP (Ferric Reducing Antioxidant Power) Method

FRAP (Ferric Reducing Antioxidant Power) assay is a method based on reducing of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) with the antioxidants' electron donation. The color change from brownish to blue is the indicator of the reaction (Rajurkar & Hande, 2011). More blue color change indicates higher antioxidant activity. Color change is detected with a spectrophotometer between 560-620 nm. Although, mostly used one is 593 nm, depending on the intensity of antioxidants, measurement can be done at higher wavelengths (Benzie & Strain, 1996). FRAP method is a very fast antioxidant assay. FRAP is a quick and simple method. It does not require highly specialized equipment or too expensive reagents (Rubio, Hernández-Ruiz, Martinez-Subiela, Tvarijonaviciute, & Ceron, 2016).

1.2.1.3 TEAC (Trolox-Equivalent Antioxidant Capacity) Method

This method is based on the reduction of $ABTS^{\bullet+}$ radical (2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid)). Firstly, $ABTS^{\bullet+}$ radical is formed with the reaction between ABTS and ferrylmyoglobin generated from metmyoglobin and

H_2O_2. Antioxidants in the environment reduce $ABTS^{\bullet+}$ radical to its nonradical form ABTS (Ilyasov, Beloborodov, Selivanova, & Terekhov, 2020). Decolorization from the typical green color of $ABTS^{\bullet+}$ is the indicator of the reaction. The color change is measured with a spectrophotometer at 734 nm. Results are calibrated with Trolox (Rubio, Hernández-Ruiz, Martinez-Subiela, Tvarijonaviciute, & Ceron, 2016). Since ABTS is a soluble radical in both organic and aqueous solvents, it can be used for determining hydrophilic and lipophilic antioxidants at the same time. However, the reaction occurs in a long period of time (Prior et al., 2005).

1.2.1.4 ABTS Cation Radical Scavenging Method

ABTS is a decolorization method based on the reduction of pre-generated $ABTS^{\bullet+}$. When $ABTS^{\bullet+}$ radical loses its typical green color, the absorption is read with a spectrophotometer at 734 nm (Charles, 2013). Results are calibrated with Trolox (Amorati et al., 2013).

This method, in fact, is an improved form of TEAC assay. In TEAC assay, early addition of the sample may result in a reaction between the antioxidants in the sample and the oxidants to form the radical. ABTS method was improved in a way that these possible interactions have been avoided. In the ABTS method, to minimize those mistakes, the sample is added after radical formation (Prior et al., 2005).

They also differ in the ways for radical formation. In the ABTS method, the radical can be formed by either different chemical or enzymatic reactions. Similar to TEAC method, the whole process requires a long time (Rubio et al., 2016).

1.2.2 Total Phenolic Content

Folin-Ciocalteu (FC) method is actually used to detect phenolic content. Because phenolic content is directly related with antioxidant property, the FC method can be explained in this section.

Folin-Ciocalteu is a colorimetric method based on the reduction of FC reagent by the phenolic compounds. Yellow FC reagent turns to blue when it is reduced under alkali conditions. The change in the color is detected with a spectrophotometer at 760 nm. Blue color intensity is proportional to the phenolic content in the sample. The calibration curve is generally prepared with gallic acid (Gülçin, 2012) .

To create the alkali environment, sodium carbonate is added to the FC and sample mixture. Therefore, color change before the addition of sodium carbonate indicates other reducing agents such as other types of antioxidants and reducing sugars. That situation is counted as the disadvantage of FC assay (Sánchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velázquez, 2013). Still, FC assay is the most commonly used method for the detection of phenolic compounds due to its simplicity, reproducibility and low cost (Charles, 2013).

1.3 Emulsions

Emulsions are colloidal systems of two immiscible fluids, which are oil and water. They form two phases, one of which is dispersed as droplets in the other (Komaiko & Mcclements, 2016). The interaction between oil and water determines the emulsion type. Dispersion of oil in water makes water continuous phase, and this type of emulsions is called oil in water (O/W) emulsions. Salad dressing, milk, mayonnaise and some beverages are typical examples of (O/W) types of emulsions in the food system. In the opposite case, oil becomes the continuous phase, and emulsion is called as water in oil (W/O) type of emulsion. Margarine is a typical food product for this class (McClements, 1999; Robins & Wilde, 2003). There are also double emulsions which can be considered as emulsions of emulsions in the

form of (O/W/O) and (W/O/W). The latter gets more attention from the food scientists because it has the potential to lower the fat content in the emulsion systems. These systems are useful regarding encapsulation of bioactive molecules (Muschiolik & Dickinson, 2017).

Emulsion systems are categorized by their droplet sizes: (conventional) emulsions, nanoemulsion, and microemulsions. With the most basic definition, conventional emulsions have the largest droplet diameters. Owing to an average droplet size of 1-2 μm, they can also be named as macroemulsions (Tadros, 2013). The use of micro and nano terms may cause some conflicts in the emulsion terminology. Although they express the exact opposite in mathematics, they were assigned to the emulsion systems in reverse (Y. Singh et al., 2017). To clarify, the acceptable average droplet size is around 300 nm for nanoemulsions, where it should be below 100 nm for microemulsions (Anton & Vandamme, 2011).

Nanoemulsions are usually known to have a milky appearance with white color (Mason, Wilking, Meleson, Chang, & Graves, 2006). They can be opaque or transparent, depending on the droplet size. Small droplets scatter the light poorly, and emulsions with smaller droplet sizes appear transparent. Therefore, nanoemulsions are expected to be more opaque than microemulsions (Molet-Rodríguez, Salvia-Trujillo, & Martín-Belloso, 2018). The opaque appearance of homogenized thyme oil can be seen in Figure 1.6.

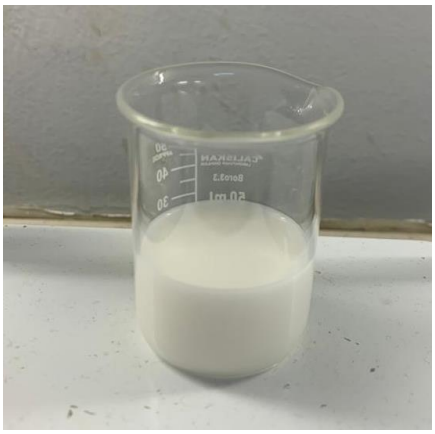


Figure 1.6: Representative photo of homogenized thyme oil emulsion

Emulsions, especially O/W types, are very common colloidal systems in the food industry. Therefore, their properties, such as quality and shelf life, have always been a hot topic for researchers. Food emulsions often suffered from lipid oxidation. The first solution to this problem is to add antioxidants to emulsions. Antioxidants' behavior in the emulsion obeys the 'Polar Paradox Theory' (Berton-Carabin, Ropers, & Genot, 2014; Choe, 2020; Shahidi & Zhong, 2011). The theory claims that polar antioxidants work more efficiently in nonpolar systems such as bulk oil compared to nonpolar antioxidants. Contrary, nonpolar antioxidants work more efficiently in relatively polar systems such as O/W emulsions. This attribution is explained by the affinity of nonpolar antioxidants towards to oil-water interface. Nonpolar antioxidants located at the interface scavenge the free radicals before they reach the lipid phase (Shahidi & Zhong, 2011). Polar antioxidants, on the other hand, locate at the water-air interface by creating a layer that protects bulk oil against oxygen (Coupland & McClements, 1996).

1.3.1 Emulsion Stability

In terms of stability, emulsions are examined in two ways: thermodynamic stability and kinetic stability. Thermodynamics of a reaction is based on the energy change between the states and controlled with Equation 1.1 where $\Delta G_{formation}$ is the difference in free energy between the initial and final stages; ΔG_{int} is the difference in interfacial free energy between initial and final states; T is the temperature and $T\Delta S_{config}$ is the configurational entropy term (McClements, 1999).

$$\Delta G_{formation} = \Delta G_{int} - T\Delta S_{config} \quad \text{(Eq. 1.1)}$$

Emulsion formation is thermodynamically unfavorable process because the change in free energy is positive. The change in interfacial free energy always yields positive because the interfacial area increases after formation. Configurational entropy also increases because there are more configurations for droplets to arrange

in the emulsified state, and disorder increases in the system. Therefore, the entropy term yields always negative. Eventually, the total free energy change for food emulsions becomes always positive due to the fact that the entropy term is much smaller than the interfacial free change for food systems (Mcclements, 2019; Tadros, 2013). A positive change in total energy is not thermodynamically favorable because molecules are prone to present in the lowest energy as much as possible.

Macroemulsions and nanoemulsions, therefore, are considered as thermodynamically unstable systems. On the other hand, during the formation of microemulsions, the entropy term dominates the interfacial free energy term, causing a negative change in total free energy. As a result, microemulsions become thermodynamically stable systems (Mcclements, 2019).

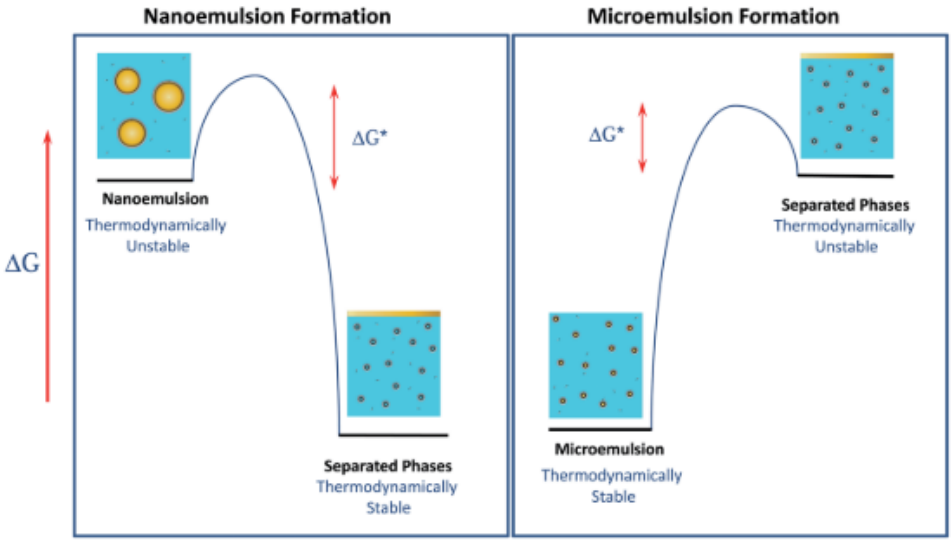


Figure 1.7: Schematic representation of two different states in nanoemulsion and microemulsion systems. ΔG^* denotes activation energy. (Mcclements, 2019)

Although emulsions will revert to the lowest energy state, they should overcome an energy barrier before that. That is the topic of kinetic stability. The more the activation energy is, the more stable emulsions are. Therefore, all emulsion types are kinetically stable for a certain time. Not surprisingly, microemulsions are the most stable ones while they are thermodynamically stable. Figure 1.7 demonstrates

the stability differences of nanoemulsions and microemulsions. Nanoemulsions have good stability with time, too, but they will break down eventually (Capek, 2004).

Emulsions break down with different mechanisms, which are given in Figure 1.8.

Sedimentation and *creaming* arise from gravity. In a gravitationally stable emulsion, while gravity favors the accumulation of the droplets, Brownian motion favors the random distribution because it increases the entropy. When Brownian motion can no longer dominate gravity, separation starts. If the droplets have a lower density than that of the surrounding medium, they move upward. If the droplets have a higher density than that of the surrounding medium, they move downward. The former is referred to as creaming while the latter is referred to as sedimentation (Tadros, 2013).

Flocculation occurs when the interaction between the droplets weaken and cannot keep them apart anymore. Droplets stick together while individual droplets are still separated. Their droplet size does not change individually; however, they form a large floc (Petsev, n.d.).

Coalescence occurs because droplets prefer to reduce the interfacial area to have the minimum energy in regard to thermodynamics. By merging, they form larger droplets. Coalescence, eventually, leads to the formation of a layer on the top of the emulsion (Capek, 2004).

Ostwald ripening (OR) is the process where large droplets become larger due to the mass transfer, while smaller droplets are disappearing. It is explained with the solubility difference of droplets due to their radius of curvatures. Therefore, OR is usually neglected in the emulsions where the oil phase is not soluble in water. In fact, it has been considered as the major destabilization mechanism in emulsions where the oil phase is relatively soluble in water. For example, when emulsions contain lipids with long chain triglycerides like sunflower oil or peanut oil, OR is not a big problem (Wooster, Golding, & Sanguansri, 2008). However, emulsions

containing partially soluble oils such as short chain triglycerides or essential oils mostly suffered from Ostwald ripening (Park, Hong, & Choi, 2020). 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 (Capek, 2004). In that regard, it can be minimized by obtaining monodisperse emulsions. However, while curvatures exist, Ostwald ripening is highly possible to occur (Tadros, 2013). Many studies have suggested that the addition of a hydrophobic compound to the oil phase is the most effective method to enhance stability against Ostwald ripening. Those compounds are called ripening inhibitors. These two types of oils are evenly distributed in the emulsion resulting in slower diffusion of the soluble one (Park et al., 2020; Wooster et al., 2008; Zhao et al., 2020).

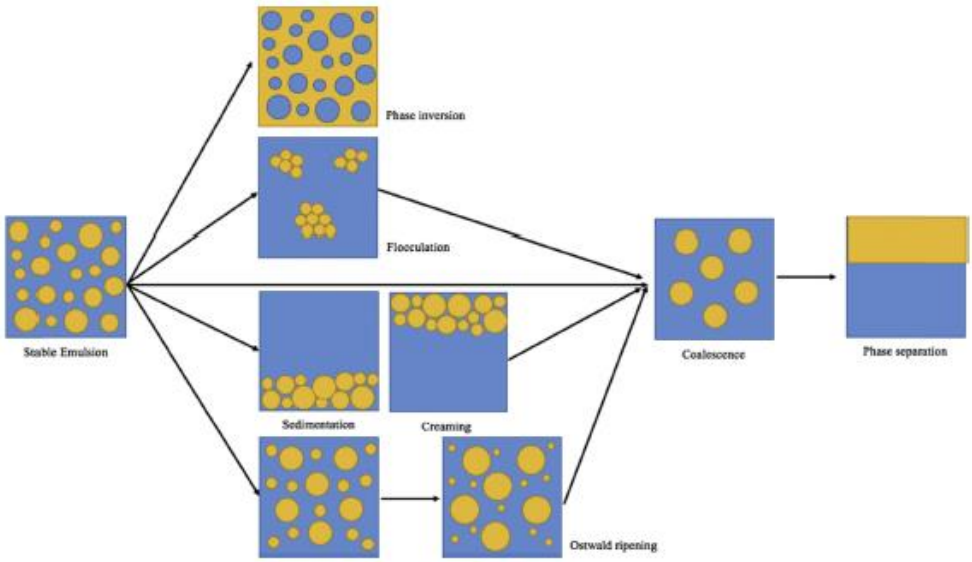


Figure 1.8: Schematic representation of destabilization mechanisms in emulsions (Hu, Ting, Hu, & Hsieh, 2017)

1.3.2 Role of Surfactants

Surfactants are surface active agents meaning they are amphiphilic compounds. Amphiphilic structure enables them to adsorb at the water-oil interface result in lowering the interfacial tension between two immiscible fluids. In that regard, a large amount of surfactant is necessary for low energy emulsification techniques. On the other hand, since high energy emulsification methods provide enough energy to achieve the energy barrier for emulsification, less amount of surfactants can be used for those kinds of emulsions (Jafari, He, & Bhandari, 2007).

Surfactant selection is very critical in emulsion systems. HLB number (hydrophilic-lipophilic balance) creates a scale for the proper selection. This numbering is the relative percentage of emulsifier's water and lipid solubility. HLB number lies between 0-20; 10 denoting equal attraction to both liquids. Surfactants with HLB number less than 10 are better for W/O emulsions. Surfactants with HLB number greater than 10 are better for O/W emulsions (Cassiday, 2016).

Surfactants can be classified according to their charge on the hydrophilic head. They can be either nonionic or ionic. Ionic surfactants with positive charged head group are cationic surfactants. Contrary, anionic ones are negatively charged. There is also another group called amphoteric surfactants. The charge of these kinds of surfactants changes depending on the pH of the environment (R. Sharma, 2014).

Tween 80 is a widely used surfactant in food, cosmetic, and drug industries. It is classified as a nonionic surfactant with HLB value of 15. Tween 80 has a small molecule that facilitates the adsorption to the interface and resulting in a stable emulsion. It is also known as being nontoxic and nonirritating. For this study, Tween 80 was used due to those advantages (Pavoni, Perinelli, Bonacucina, Cespi, & Palmieri, 2020). The typical structure of Tween 80 is given in Figure 1.9.

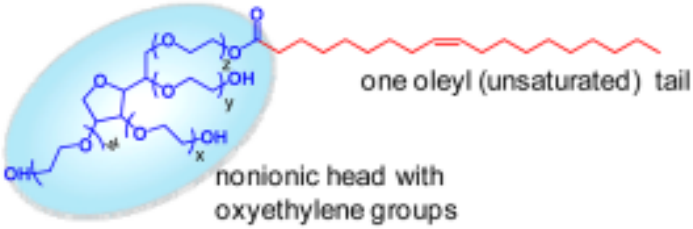


Figure 1.9: The molecular structure of Tween 80, blue part denotes hydrophilic head and red part denotes hydrophobic tail (Athas et al., 2014)

1.3.3 Emulsification Techniques

Emulsification techniques can be classified into two groups as high energy techniques and low energy techniques. While high energy methods exert the high shear forces, low energy methods are controlled by the physiochemical interactions between the surfactants and liquids (Santana, Perrechil, & Cunha, 2013). High energy methods are widely used for industrial purposes rather than low energy methods. From an economic perspective, high energy methods are more costly; however, they are more preferable in industry. Because it is easier to control the size of the droplets with the parameters in high energy equipment. Moreover, they require less amount of time. It is possible to form more stable emulsions with smaller droplets by using less amount of emulsifier when compared to low energy methods (Saffarionpour, 2019).

1.3.3.1 High Energy Methods:

1.3.3.1.1 Ultrasonication

A sonic probe achieves ultrasonic homogenization. When the tip is dipped into the coarse emulsion, ultrasonic waves create acoustic cavitation and vibration, resulting in the formation of air bubbles in the emulsion (Taha et al., 2020). When

air bubbles cannot expand further, they collapse by creating shock waves and turbulence. This action, which is demonstrated in Figure 1.10, is enough for large droplets to break into small droplets (Kentish et al., 2008). Sonication can be used on small scales because it is not suitable to produce large volumes of nanoemulsions with this probe application (Jasmina, Dzana, Alisa, Edina, & Ognjenka, 2017).

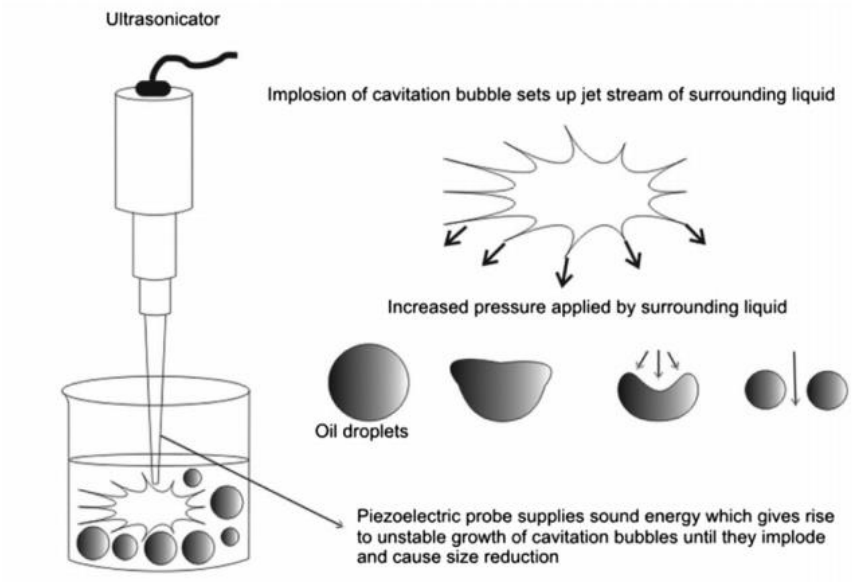


Figure 1.10: Schematic representation of ultrasonication method (Cheaburu-Yilmaz, Karasulu, & Yilmaz, 2018)

1.3.3.1.2 High Pressure Homogenization

High pressure homogenizers are one of the most used methods to produce nanoemulsions with fine droplets. It is common in both industry and laboratory scales (Jasmina et al., 2017). It creates disruptive forces such as cavitation, shear, and turbulence, by applying high pressures. The coarse emulsion is fed into the homogenizer and passed through a narrow orifice. With the forces occurred due to

pressure, big droplets are disturbed and break into small droplets (Saffarionpour, 2019). The mechanism of high pressure homogenizers is shown in Figure 1.11.

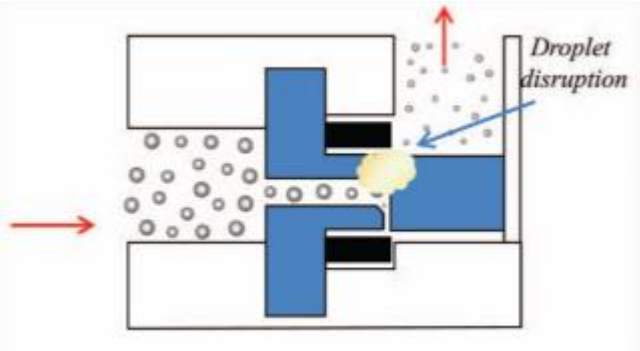


Figure 1.11: Schematic representation of high pressure homogenizers (McClements & Rao, 2011)

1.3.3.1.3 Microfluidization

Similar to the high pressure homogenizers, microfluidizers exert high pressure, as well. It can reach up to 150 MPa (1500 bar) (Jafari et al., 2007). With a high power pump, the coarse emulsion is forced through channels which can be seen in Figure 1.12. This channel is split into two channels that enable these two streams to impinge with each other (McClements, 2011). Disruptive forces that arise from the collision under high pressures are enough to obtain small droplets. With this method, emulsions with 100 nm – 1000 nm droplet size can be formed. By increasing the number of cycles, both small droplets and narrow distribution can be achieved (Vladisavljević, 2018).

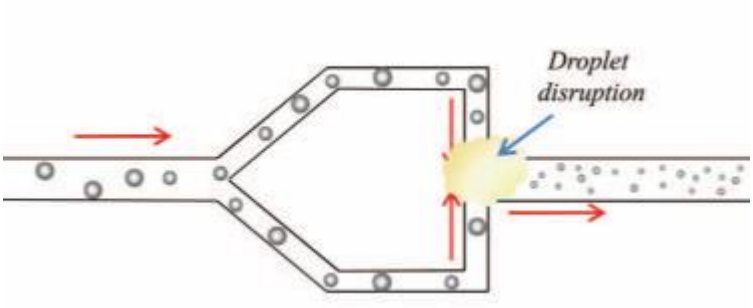


Figure 1.12: Schematic representation of microfluidizers (McClements & Rao, 2011)

1.3.3.2 Low Energy Methods:

1.3.3.2.1 Spontaneous Emulsification

It is also known as self-emulsification. This technique is based on pouring the phase containing surfactant into the other phase at a constant temperature. For example, when an organic phase consisted of oil and a hydrophilic surfactant is mixed with water, the surfactant tries to move towards the aqueous phase. This movement creates an oil-water interfacial area and eventually leads to oil droplets surrounded by water droplets (Santana et al., 2013). The mechanism can be seen in Figure 1.13.

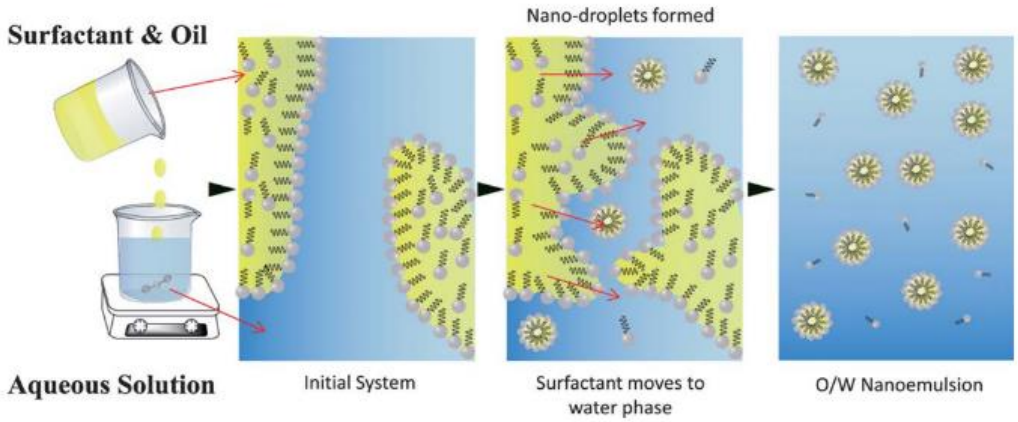


Figure 1.13: Schematic representation of spontaneous emulsification mechanism (McClements, 2011)

1.3.3.2.2 Phase Inversion Temperature

Phase inversions temperature is based on the solubility change of a nonionic surfactant with temperature. In such systems, a transition occurs from W/O emulsion to O/W emulsion or the opposite. The temperature where the transition takes place is called PIT (Phase inversion temperature) (McClements, 2011). At the beginning before the PIT, surfactant behaves hydrophilic and favors O/W emulsions. With the increasing temperature, the solubility of the surfactant in water decreases, and it becomes more soluble in lipid. With this hydrophobic effect, hydrophilic head groups of surfactant molecules come together, and they form a curvature favoring W/O emulsion (Jasmina et al., 2017). The mechanism can be seen in Figure 1.14.

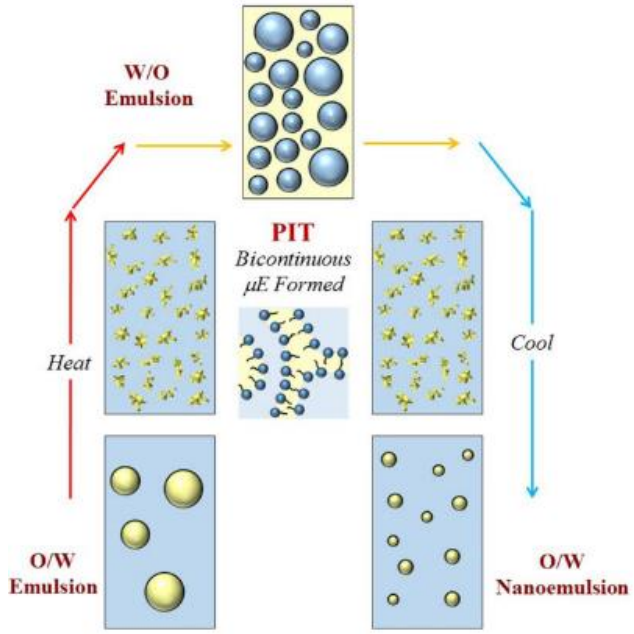


Figure 1.14: Schematic representation of emulsification with phase inversion temperature (Komaiko & Mcclements, 2016)

1.3.4 Characterization of Emulsions

Characterization of homogenized emulsions includes both physical and chemical tests. Droplet size, zeta potential, viscosity, density, stability, pH, or conductivity can be examples of characterization ways. Some of these techniques are discussed below:

1.3.4.1 Droplet Size

1.3.4.1.1 Dynamic Light Scattering (DLS)

Droplet size is the most important parameter while forming homogenized emulsions. The technique uses Brownian motion theory, which is the random movement of the droplets due to collisions in the medium. When a solution is faced

with a beam of light, light scatters in all directions, and a detector records the intensity of the scattered light (Sandhu, Singh, Dhankhar, Kama, & Sharma, 2018). Scattered light fluctuates with time depending on the droplet size. Fluctuations can be seen in Figure 1.15. Small molecules diffuse faster and they result in faster fluctuations (Stetefeld, McKenna, & Patel, 2016). The intensity of fluctuations is correlated with diffusion coefficient which is a parameter in Stokes-Einstein equation. The following equation relates the droplet size and diffusion coefficient:

$$d_H = \frac{kT}{3\pi\eta D} \qquad \text{(Eq. 1.2)}$$

Where D is the diffusion coefficient; d_H is the hydrodynamic diameter; k is Boltzmann's constant; T is the temperature, and η is the viscosity.

Samples usually are diluted to avoid multiple scattering effects.

DLS reports the droplet size and the polydispersity index. PDI lies between 0 and 1, where 0 denotes monodisperse systems and 1 denotes polydisperse systems (Gurpreet & Singh, 2018).

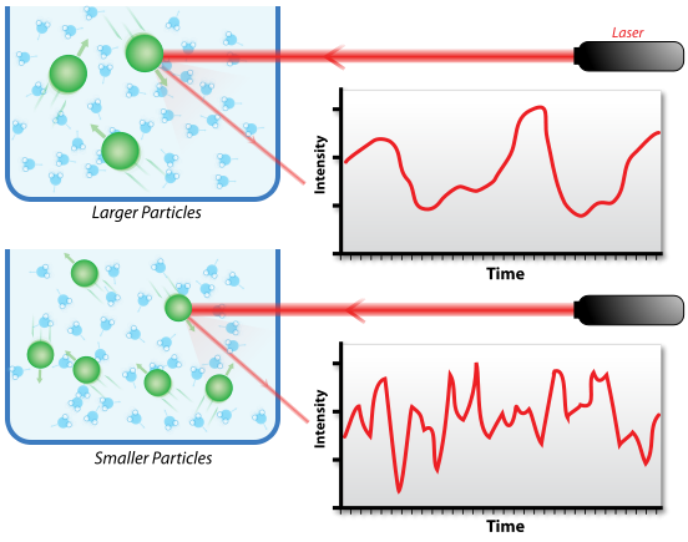


Figure 1.15: Fluctuations of two different sized droplets in DLS

1.3.4.1.2 Transmission Electron Microscopy (TEM)

There is an analogy between light microscopes and electron microscopes. However, an electron beam illuminates the specimen in electron microscopes rather than a light beam, as in the light microscopes. A drop of the sample is poured on a grid; then, it is stained with a heavy metal salt solution and left to dry. Following, electrons are accelerated with voltage in a vacuum. Since the electrons cannot pass through metal, they form a contrasting image while overshadowing the droplets (Klang, Matsko, Valenta, & Hofer, 2012; Kuntsche, Horst, & Bunjes, 2011). TEM creates a 2D image with a high resolution.

Droplet size analysis with TEM is mostly applied after having the result with the other techniques so that the results become in accordance. Besides the size, they provide morphological information about the sample, too. TEM has too many advantages. They give very detailed information about the structure, surface, size, or shape. Images obtained with TEM have very good resolutions. They can be used for a very wide range of applications. Since the operation is conducted under vacuum, only vacuum tolerated samples can be imaged (Choudhary & Choudhary, 2018).

1.3.4.2 Zeta Potential

In colloidal systems, zeta potential gives the difference in potential between the dispersed and the continuous layers in that dispersed droplet. Zeta potential is also known as electrokinetic potential. The magnitude of zeta potential has been used as an indicator of stability (Lu & Gao, 2010). Outside of the range between -30 mV and +30 mV, zeta potential represents stable systems. Because when all droplets have the same large potential, they will repel each other. Therefore, emulsions show good stability because repulsive forces dominate attractive forces. However, when zeta potential approaches zero, interactions between droplets become easier due to the lack of surface charge (Seibert et al., 2019). Therefore, emulsions tend to

break down quickly. pH is the most important factor in zeta potential. Besides pH, it is also affected by concentration and temperature (Lowry et al., 2016).

1.3.4.3 Time Domain Nuclear Magnetic Resonance (TD-NMR) Relaxometry

TD-NMR is one of the most popular characterization techniques in the food industry. It is also known as low field NMR. Being simple, quick, and portable makes TD-NMR a preferred analysis. The most significant advantage of NMR is that it is a nondestructive method, meaning during the measurement sample is not affected physically or chemically (Marcone et al., 2013).

It is based on the observation of the changes in the alignment of the protons when they are exposed to a radiofrequency (RF) pulse. Naturally, protons are oriented randomly, but when they are exposed to a magnetic field, they line up and start to precess. With the external magnetic field generated by NMR equipment, protons start to precess about z axis being out of phase with each other. When an RF pulse is transmitted, protons are flipped down to the x-y plane. This time they precess in phase. When the RF pulse is removed, protons start to turn back their previous states. This turning back is called relaxation (Hashemi, Bradley Jr., & Lisanti, 2012). NMR measures two types of relaxation time. Longitudinal relaxation time, T_1 is time for the protons to realign themselves in the z-axis. This is also called spin-lattice relaxation time because protons turn back to their lowest energy states by giving the excess energy to the surrounding lattice (Parlak & Guzeler, 2016). Transverse relaxation time, T_2 is much more related to spin-spin interactions. It is the time indicating the rate of the decay of the magnetization on the x-y plane. T_1 and T_2 relaxation time are intrinsic properties of the materials. Their use is mostly associated with the identification of oil and water compartments in emulsions. It is a fact that water has a very long T_1 and T_2 times and oil has shorter T_1 and T_2 times (Hashemi et al., 2012; Kirtil & Oztop, 2016). Figure 1.16 and Figure 1.17 show typical example of T_1 and T_2 relaxation times, respectively.

In emulsion systems, NMR puts one more advantage to common methods. NMR is able to measure viscosity or droplet diameter of the non-transparent emulsions without making any dilution while it is a must in other methods such as dynamic light scattering or transmission electron microscope (Kirtil, Cikrikci, McCarthy, & Oztop, 2017).

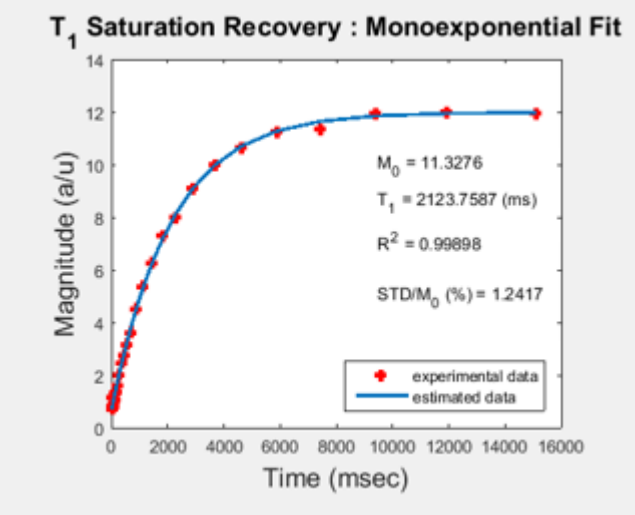


Figure 1.16: A representative T1 recovery curve

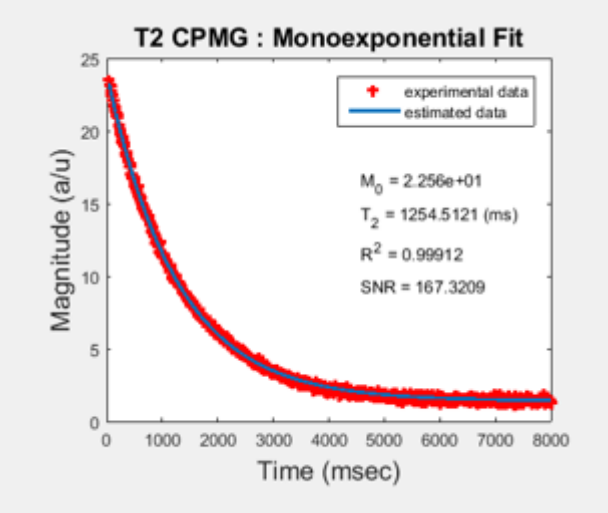


Figure 1.17: A representative T2 decay curve

1.3.5 Objective of the Study

This study aims to formulate and characterize emulsions with clove and thyme essential oils. Specific objectives for this purpose can be listed as follow:

- To understand the effect of essential oil type and concentration on the droplet size of emulsions
- To understand the effect of essential oil type and concentration on the stability of emulsions
- To understand the effect of microfluidization on the particle size and stability of emulsions
- To understand the effect microfluidization on the antioxidant capacity of emulsions
- To see whether NMR relaxometry can be used as a method for emulsion characterization

CHAPTER 2

MATERIAL AND METHODS

2.1 Materials

Both clove oil and thyme oil were purchased from Botalife (Isparta, Turkey). Tween 80, ethanol, methanol, acetic acid, sodium hydroxide, Folin-Ciocalteu reagent, and gallic acid were obtained from Merck (Darmstadt, Germany). Trolox, DPPH and TPTZ reagents, sodium carbonate, ferric chloride and sodium acetate trihydrate were Sigma-Aldrich products (St. Louis, MO, USA).

2.2 Methods

2.2.1 Sample Preparation

Emulsions were prepared by mixing essential oil as the dispersed phase and distilled water as the continuous phase. In emulsions, clove oil and thyme oil were used as essential oils. Tween 80 was selected as the surfactant. Emulsions were formulated with two different essential oils in two different ratios (2% v/v and 4% v/v). Each of them was prepared with a 1:1 surfactant to oil ratio, the rest being distilled water.

2.2.1.1 Preparation of Primary Emulsions

The essential oil-surfactant-water mixture was pre-homogenized with Ultra-Turrax (WiseTis homogenizer, Witeg Labortechnik GmbH, Germany). This treatment lasted 2 min at 10000 rpm.

2.2.1.2 High Pressure Homogenization of Emulsions

Pre-homogenized emulsions were fed into a microfluidizer. They were exposed to ~1300 bar (130 MPa) for five cycles in Nano-Disperser (NLM 100, South Korea). Homogenized emulsions were collected for further analyses.

Samples were abbreviated according to their essential oil composition (clove (C) vs thyme (T)) and homogenization method type (high shear (P) vs microfluidization (H)). The number denoted the concentration of essential oil in the formulation. For example, 'C2-P' denoted an emulsion with 2% of clove oil prepared by high shear homogenization.

2.2.2 Characterization of Emulsions

2.2.2.1 Droplet Size and Zeta Potential Analysis

The droplet size of the primary emulsions and homogenized emulsions was measured every two weeks in one month period. These measurements were conducted with the Malvern Zetasizer instrument (Nano ZS90, Worcestershire, UK) found in METU Central Laboratory. Before each measurement, all samples were diluted to 1:100 ratio with distilled water to prevent possible multiple scattering effects. Additionally, PDI (Poly Dispersity Index) of the results was recorded. Zeta potential was measured only for homogenized emulsion samples.

2.2.2.2 Transmission Electron Microscopy (TEM)

For morphologic analysis and to confirm the droplet size results from DLS, TEM analysis was conducted. The images were collected from Transmission Electron Microscobe (Tecnai G^2 Spirit Biotwin, FEI Company) at METU Central Laboratory. A drop of the emulsion was poured into the copper grid. It was let to dry at room temperature. Finally, the contrast was created with the electrons accelerated in 120 kV.

2.2.2.3 Antioxidant Capacity

Antioxidant capacity and total phenolic content were investigated for this section. All measurements were applied to both primary and homogenized emulsions and pure essential oils.

2.2.2.3.1 Antioxidant Capacity by DPPH Method

Antioxidant capacity of the emulsions was measured spectrophotometrically according to DPPH method described by Zhang, Guo, Guo, Jiang, & Ji (2018) with some modifications. First, DPPH solution was prepared by dissolving 5 mg of DPPH reagent in 200 ml of methanol. 0.1 g of emulsions were dissolved in 1 ml mixture of ethanol: acetic acid: water (50:8:42 v/v). After a 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 one hour in the dark cabinets. When the incubation time was over, absorbance values were recorded at 517 nm with UV Spectrophotometer (Optizen Pop Nano Bio, Korea). The same procedure was applied to the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solution and calibration curve was plotted with Trolox concentration against the absorbance values. Calibration curve is given in the

Appendix A. The results were expressed as μmol of Trolox per gr of sample (μmol Trolox/gr sample).

2.2.2.3.2 Antioxidant Capacity by FRAP Method

Antioxidant capacity of the emulsions was measured spectrophotometrically according to the FRAP method described by Benzie & Strain (1996) with minor modifications. 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 water containing 8 mL of glacial acetic acid. The pH of the mixture was adjusted to 3.6 with 1 M sodium hydroxide. Mixture is completed to 500 mL with water. TPTZ stock solution was prepared by dissolving 156 mg of 2,4,6 tripyridyl-S-triazine (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 dark for 4 minutes. As the reaction time is reached, absorbance at 593 nm was recorded with a UV Spectrophotometer (Optizen Pop Nano Bio, Korea). The same procedure was applied to the Trolox solution and the calibration curve was plotted with Trolox concentration against the absorbance values. Calibration curve is given in the Appendix A. The results were expressed as μmol of Trolox per gr of sample (μmol Trolox/gr sample) (Rajurkar & Hande, 2011).

2.2.2.4 Total Phenolic Content

Total phenolic content of the emulsions was measured spectrophotometrically according to Folin-Ciocalteu method explained in (Moisa et al., 2018) with minor modifications. FC (Folin-Ciocalteu) reagent was diluted with distilled water (1:100) (v:v). 20 μl of emulsion was poured into 2 mL of diluted reagent.

Following shaking and three minutes, 700 μL of saturated sodium carbonate solution was pipetted into the previous mixture. The final mixture was kept at room temperature for one hour in dark cabinets. Finally, the color change was detected with UV Spectrophotometer at 760 nm. The same procedure was applied to the gallic acid solutions to draw calibration curve which is given in the Appendix A. The results were expressed as mg of Gallic Acid Equivalent per gr of sample (mg GAE/gr sample).

2.2.2.5 Time Domain Nuclear Magnetic Resonance (TD-NMR) Relaxometry

NMR experiments were performed in a bench-top NMR system operating at a ^{1}H frequency of 20.34 MHz (Resonance Systems GmbH, Germany). T_1 and T_2 relaxation time measurements were carried out to pure essential oils, primary and homogenized emulsions. To detect T_2 times, CPMG sequence was run with the following parameters: echo time of 10000 μs; 800 echoes and 4 scans. To detect T_1 time, saturation recovery pulse sequence was used with the parameters: relaxation period (TR) of 500 ms and 4 scans. Observation time was between 10 ms and 15061 ms.

2.2.2.6 Instantaneous and Long Term Stability Tests

Stability tests were done only for homogenized emulsions since primary emulsions are prone to break down easily. They were stored at room temperature.

Instantaneous stability was checked by putting the samples into the high speed mini centrifuge (MicroSpin12, USA). After pouring 1 ml of samples into mini tubes, the height was recorded. They were subjected to 15115 x g for 1 minute. Finally, in case of any phase separation, the height of the separation was recorded. The ratio between the initial and final heights were reported as ‘instantaneous stability’ (Kumar et al., 2015).

For long term stability, homogenized emulsions were first observed with the naked eye. Emulsions with any visual separation were not tested further. The ones without any visible problem were tested with the same centrifuge method. Long term stability tests were applied for three months.

2.2.2.7 Viscosity Measurements

Viscosities of pure essential oils were measured with the vibro viscometer (SV10, A&D Company) to understand their behaviors in emulsions and to interpret TD-NMR relaxation times.

2.2.3 Experimental Design

Experimental design is given in the Table 2.1

Table 2.1: Experimental design with factor, levels and responses

Factors	**Levels**	**Responses**
Essential oil type	Clove oil Thyme oil	1. Determination of Droplet Size 2. Determination of Zeta Value
Essential oil concentration	2% 4%	3. Quantification of Antioxidant Capacity 4. Quantification of Total Phenolic Content
Homogenization method	High shear homogenization (HSH) Microfluidization (MF)	5. Stability Observations 6. TEM Analyses 7. TD-NMR Relaxometry Analyses

2.2.4 Statistical Analysis

Statistical analyses were performed by using analysis of variance (ANOVA) with Minitab V19 (Minitab Inc, Coventry, UK). Tukey's test was used for comparison with a confidence level of 95%. Correlation tests were applied for the antioxidant capacity and total phenolic content measurements. Box-cox transformation was applied when it was necessary. All experiments were conducted with three replicates. Different letters denote significant differences among the samples ($p<0.05$).

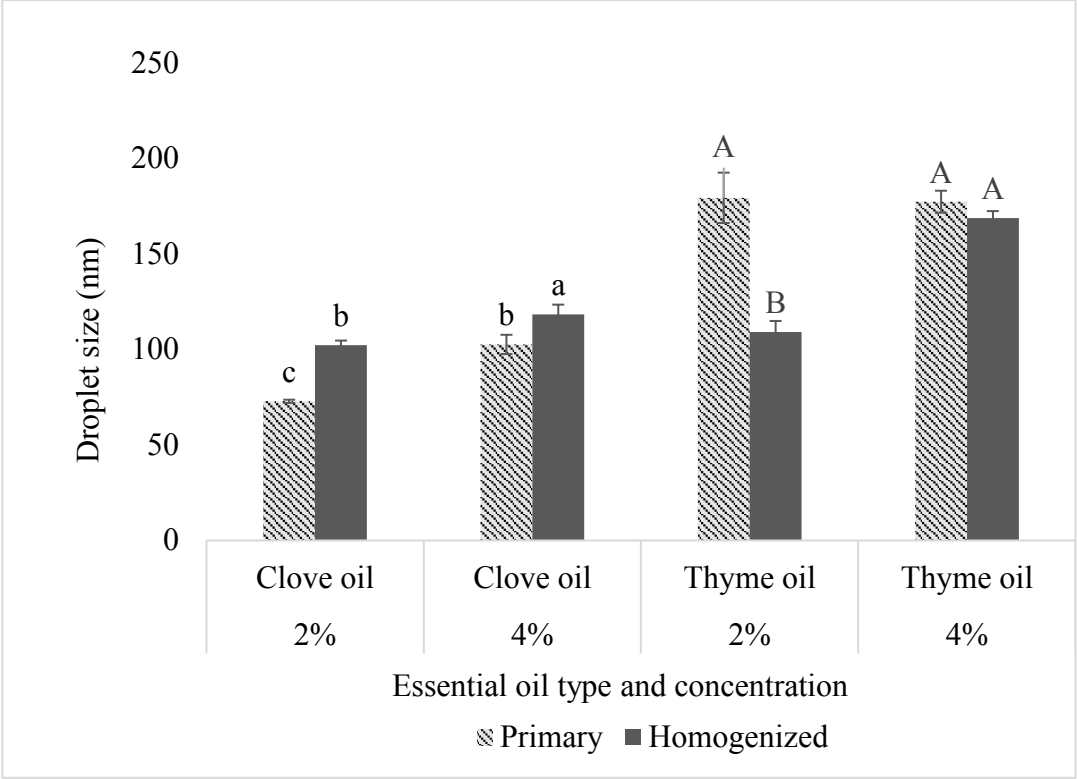
CHAPTER 3

RESULTS AND DISCUSSION

3.1 Droplet Size of Emulsions

Droplet size is the most important criteria to check in emulsion systems. The result of droplet size measurements identifies the emulsion type and also gives an idea about stability when measured over time. Droplet sizes of fresh emulsions are given in Figure 3.1. The results are in accordance with the literature. It has been reported that clove oil nanoemulsions prepared with microfluidization had approximately 100 nm droplet (Wan, Zhong, Schwarz, Chen, & Rao, 2018). In another study, Moradi & Barati (2019) reported a range of 180-200 nm for droplet sizes for emulsions prepared with different thyme oil concentrations.

It is clear that clove oil emulsions gave smaller droplet size results than thyme oil emulsions. Oil type and surfactant interaction might be the reason for this difference. A study where microemulsions composed of essential oils were compared revealed that Tween 80 was more efficient in clove oil emulsions than thyme oil emulsions (Edris & Malone, 2012). They hypothesized that surfactant like structure of eugenol increased the surfactant efficiency. It was stated earlier in the Introduction section that both eugenol and Tween 80 had a double bond in their structures. Interaction between those double bonds caused better adsorption of Tween 80 in clove oil emulsions. Similar results were also obtained in the study where clove oil was nanoencapsulated (Nagaraju, Sengupta, Priyadarshini, & Rao, 2020). They found out that Tween 80 easily adsorbed around the clove oil droplets due to the presence of double bond on its nonpolar chain. To conclude, better adsorption decreased the interfacial tension at a higher rate and resulted in droplets with smaller sizes.



Different small letters indicate significant differences among clove emulsions; Different capital letters indicate significant differences among thyme emulsions

Figure 3.1: Droplet size results of all emulsion types on the first day

3.2 Transmission Electron Microscopy Results

According to Figure 3.2, it can be seen 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 quite expected because drying of samples for TEM analysis may cause shrinkage (Klang et al., 2012).

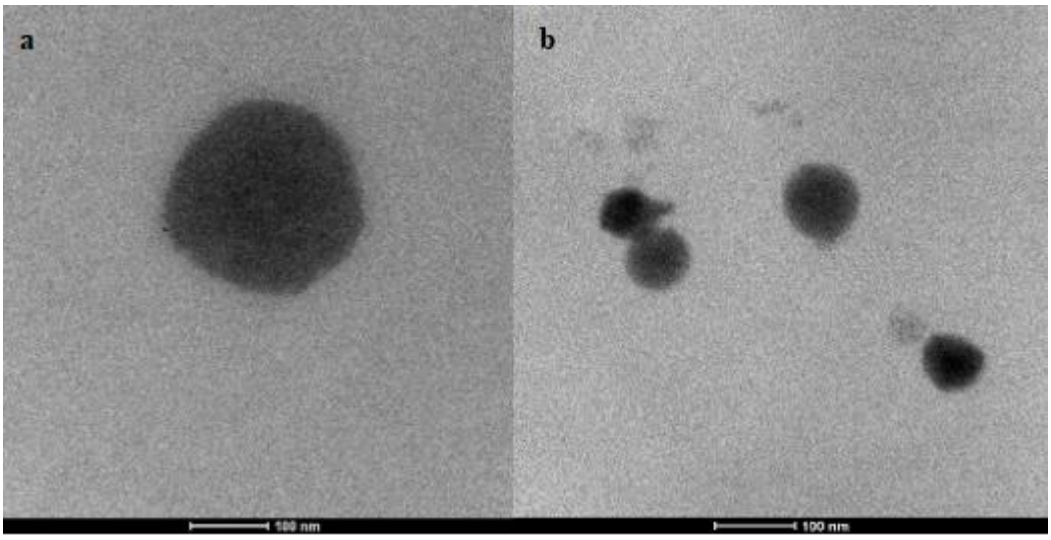


Figure 3.2: Transmission electron micrographs of primary (a) and homogenized (b) thyme oil emulsions with a scale of 100 nm

3.2.1 Microfluidization Effect on Droplet Size and PDI Values

High pressure homogenization (microfluidization) was applied to emulsions prepared by using a high shear mixer. This process not only aims to produce smaller droplet size emulsions but also is expected to decrease PDI and both of these changes are expected to increase the stability of the emulsions. However, this was not the case observed for the essential oil emulsions produced in this study. As seen in Figure 3.1, microfluidization did not decrease the droplet size except 2% thyme oil emulsions. In fact, 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 enables to obtain emulsions with fine droplets thanks to its controllable parameters, pressure, and number of cycles. By increasing both parameters, it is expected to form much smaller droplets since disruptive forces on droplets increase (Uluata, Decker, & McClements, 2016). 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 called ‘recoalescence’. The emulsification process should be examined here. Emulsification consists of several steps: Disruption of droplets, covering of freshly formed interfaces by emulsifier, collisions, and possible coalescences happen very quickly in milliseconds. Therefore, droplet size reduction is known as the balance between droplet break up and recoalescence (Tang, Shridharan, & Sivakumar, 2013). Actions of emulsifier, amount of energy supplied to the system, dispersed phase properties and concentration, viscosity, temperature are the factors that control the droplet size reduction. To obtain a stable emulsion with fine droplets and no recoalescence, all these factors with optimal conditions should be arranged (Stang, Schuchmann, & Schubert, 2001). Figure 3.3 shows a relationship between droplet size of the emulsion and the dispersed phase concentration with homogenization pressure according to the study conducted with orange essential oil.

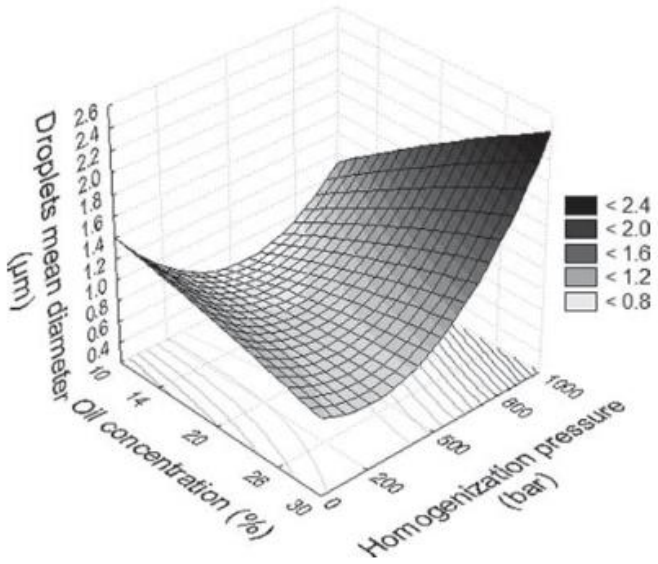


Figure 3.3: A graphical demonstration of the relationship between droplet size and dispersed phase concentration and homogenization pressure (Carmona, Tonon, Cunha, & Hubinger, 2013)

The actions of emulsifiers are critical in many ways. For example, if there is not enough emulsifier, not all droplets can be covered by the emulsifier, and they stick with their neighbors. Additionally, the emulsifier should adsorb and stabilize the

droplets in a very short time. If stabilization is achieved slower than collusions, then recoalescence starts (Jafari, Assadpoor, Yinghe, & Bhandari, 2008).

Energy input is another critical parameter. There is such a pressure value that the droplet size cannot be reduced further after that point. If extra energy is supplied to the system by applying unnecessarily high pressures, 'overprocessing' might happen and cause recoalescence (Santana, Perrechil, & Cunha, 2013a). Therefore, 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 (Floury, Desrumaux, Axelos, & Legrand, 2003; Santana, Perrechil, & Cunha, 2013b). Overprocessing was observed in these studies above the optimum pressures.

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. Similarly, collision frequency is the number of collisions per unit volume and unit time. Not surprisingly, as the probability of collision and its frequency increases, coalescence increases (Jafari et al., 2007). Collision frequency is highly affected by the viscosity, energy input, and droplet size. The faster the droplets in the channels of high pressure homogenizers, the more collisions take place. Droplets may reach high velocities when emulsion has low viscosity or when they are given extra energy. If they are small droplets, they speed up even more. They are all related to the mobility of the droplets. Especially viscosity determines the mobility (Tesch & Schubert, 2002).

At this point, a viscosity comparison between essential oils and vegetable oils can enlighten the problem better. Sunflower oil was selected as the vegetable oil representative because its use is more often. The viscosity of sunflower oil is measured as 63 cP (at 20°C) (Calligaris, Mirolo, Pieve, Arrighetti, & Nicoli, 2019). In this study, the viscosity of pure oils was measured and it is given in Table 3.1. As it is seen, 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. When extra energy is given, they may suffer from the recoalescence due to high mobility arose from low viscosity. A study conducted on emulsification with high pressure homogenizers suggest that there is no need to use developed high pressure homogenizers for low-viscosity emulsions because they can already be produced with low pressures (Stang et al., 2001). They add further that when those homogenizers are used at high pressures, they even adversely affect droplet size due to overprocessing.

Table 3.1: Viscosity results of essential oils

	Temperature (°C)	**Viscosity (cP)**
Distilled water	17.7	1.14
Thyme oil	17.1	15.5
Clove oil	17.0	9.03

To conclude, what happened with the clove oil emulsions in this study is an example of 'overprocessing'. Due to its low viscosity, droplets of clove oil emulsions accelerated too much in the channels. These results are similar to those reported in a high pressure homogenization of orange essential oil emulsion. The authors observed an increase in droplet size after the emulsification process (Carmona et al., 2013). Actually, what they did was to understand the pressure effect by applying different pressure values for 1 cycle. They showed that below 500 bar droplet size decreased. However, higher pressure values than 500 bar caused droplet size to increase. Researchers tried to find optimum conditions for microfluidizer and proper selection of surfactant for d-limonene emulsions (the major component of citrus essential oils) (Jafari et al., 2007). They applied different pressures with 2 cycles. Up to a certain pressure limit, the droplet size did not change at all. When higher pressures were applied, they observed coalescence

and an increase in the droplet size. They explained this problem with the low viscosity of d-limonene and poor stabilization of Tween 20. They also examined the different surfactant types. They reported that surface active biopolymers were better choices for obtaining smaller droplets and more extended stability. Because, in their case, low viscosity was compensated with a thicker surfactant. As a conclusion, the mobility of droplets can only be controlled with a proper emulsifier selection because oil and water phases are fixed in the formulations. Especially in cases where the viscosity of components is low, emulsifier plays a critical role (Tang et al., 2013).

As seen in Figure 3.1, microfluidization caused a significant reduction in the droplet size in 'T2-P' ($p<0.05$). It can be concluded that conditions of homogenization process worked most successfully with 2% thyme oil emulsions. On the contrary, no change in the droplet size was observed in 4% thyme oil emulsion. The problem with that sample was thought to be the lack of 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. Probably, droplet size first decreased however, surfactant could not cover all droplets, and they coalesced with the surrounding droplets (Jafari et al., 2007). Finally, droplet size remained unchanged.

PDI results are given in Table 3.2. Microfluidization was sufficient for reducing PDI in all emulsions. This result is similar to the findings reported by the study with cinnamon oil emulsification. Researches indicated that PDI value of emulsions decreased after high pressure homogenization (Aisyah, Haryani, Safriani, & Husna, 2018). This reduction was an expected result because emulsification processes homogenize the emulsions, and therefore polydispersity index decreases (Clayton, Salameh, Wereley, & Kinzer-Ursem, 2016).

Table 3.2: PDI values of emulsions

Sample ID	PDI	Sample ID	PDI
C2-P	0.410 ± 0.007^{a}	**T2-P**	0.385 ± 0.002^{b}
C2-H	0.211 ± 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}

PDI values were compared separately for clove and thyme oil emulsions

3.3 Zeta Potential Analysis

Zeta potential analysis was only conducted on homogenized samples. The results were as follows: 'C2-H': -14.8 mV, 'C4-H': -15.9 mV, 'T2-H': -11.2 mV, and 'T4-H': -17.4 mV. It is usually believed that when zeta value lies between -30 mV and 30 mV, that system is not stable (Lu & Gao, 2010). Given the meaning of zeta potential range, these values indicate that none of them are stable. In fact, there are many studies stating that there is no correlation between zeta value and stability in their systems (Aida, Mustapha, & Mohamad, 2018; Noori, Zeynali, & Almasi, 2018; Roland, Piel, Delattre, & Evrard, 2003; Seibert et al., 2019).

Zeta potential is the electronic potential on the double layer. With the most basic term, it can be considered as the surface charge on the layer. Therefore, zeta potential justifies the stability with the presence of repulsive forces (Lu & Gao, 2010). However, it is not valid for all systems. It is known that nonionic surfactants stabilize the system with steric repulsion. Among the nonionic surfactants, Tween 80 is a widely used one. For example, in this study, one could expect to obtain a zeta value close to zero due to Tween 80. However, it is not the case. Noori et al. (2018) found out similar findings in their nanoemulsions prepared with ginger essential oil and Tween 80, and they explain it with the presence of ionizable groups in essential oils. According to their study, mechanical stress occurring during homogenization may cause dissociation of hydroxyl groups in essential oils

(Artiga-Artigas, Acevedo-Fani, & Martín-Belloso, 2017). Deprotonation creates a negative charge. Consequently, it yields a negative zeta potential. Depending on the dissociation degree, each essential oil may give different zeta potential values in the same conditions (Bonilla, Atarés, Vargas, & Chiralt, 2012).

As explained in detail, the zeta potential results in this study did not provide information about the stability. Although the results were similar in emulsions with both clove and thyme oils, the latter exhibited way better stability, which will be discussed later.

3.4 Antioxidant Capacity

3.4.1 Antioxidant Capacity of Pure Essential Oils by DPPH and FRAP methods

In DPPH method, while pure clove oil showed 1213.8 ± 5.2 μmol Trolox/gr oil, thyme oil showed 11.3 ± 0.1 μmol Trolox/gr oil antioxidant capacity. In FRAP method, while pure clove oil showed 1734.5 ± 110.7 μmol Trolox/gr oil, thyme oil showed 13.9 ± 0.2 μmol Trolox/gr oil antioxidant capacity. The results of both methods were consistent with each other.

Wang, Yih, & Yang (2017) reported the antioxidant capacity of clove oil as 809 ± 0.01 μmol Trolox/gr oil in their study. In another study, antioxidant capacity of oregano oil was found as 20.99 ± 1.24 μmol Trolox/gr oil (Simirgiotis et al., 2020). The result is acceptable since oregano oil and thyme oil have very similar structures. They are both composed of thymol and carvacrol (Tavakoli et al., 2017).

Clove oil has been identified as the most powerful antioxidant in many comparative studies conducted with BHT, BHA, and Trolox (Arenas, Amner, Mendez, & Kouznetsov, 2011; Gulcin, 2014). Besides, among the different essential oils, clove oil has been reported as the strongest one (Aktas, Ozdemir, & Basmacioglu, 2018; Ghadermazi, Amir, & Goli, 2017; Politeo, Juki, & Milo,

2006). Authors compared the antioxidant capacity of thyme and clove oil in their study, and clove oil turned out to be 70 times more powerful antioxidant than thyme oil (Zengin & Baysal, 2014). In addition, Viudamartos et al. (2010) compared the five different essential oils, including clove and thyme oil, and they found out that clove oil exhibited the highest antioxidant capacity.

3.4.2 Comparisons and Correlation Between Antioxidant Activities of Emulsions Determined with DPPH and FRAP Assays

3.4.2.1 Effect of Microfluidization on Antioxidant Capacity

Emulsions were exposed to high pressure (~130 MPa) with a microfluidizer. Antioxidant activities of primary and homogenized emulsions were compared. As seen in Table 3.3 and Table 3.4, according to the DPPH method results, microfluidization did not affect emulsion's antioxidant capacity ($p>0.05$). Primary and homogenized emulsions showed the same antioxidant capacity both in clove and thyme oil samples. The result is important because after such mechanical exposure, essential oils kept their antioxidant capacity.

The results of FRAP assay are given in Table 3.5 and Table 3.6. FRAP method gave similar results only with one exception. Microfluidization appears to cause a decrease only in C4-P sample. Other emulsions seem unaffected ($p>0.05$). The difference between DPPH and FRAP method arises from the fact that they have different mechanisms. As mentioned earlier, free radicals are neutralized either by electron or hydrogen atom transfer in 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}. As understood, chemicals used and the environment are also quite different from each other (Liang & Kitts, 2014). To conclude, mechanical stress induced by high pressure may have resulted in different antioxidant values for different methods.

Table 3.3: Antioxidant capacity of clove oil emulsions determined with DPPH assay

Sample ID	Concentration	Emulsion type	Antioxidant Capacity (µmol Trolox/gr emulsion)
C2-P	2	Primary	12.02 ± 0.08^{b}
C2-H	2	Homogenized	11.98 ± 0.06^{b}
C4-P	4	Primary	120.51 ± 0.52^{a}
C4-H	4	Homogenized	118.71 ± 1.56^{a}

Table 3.4: Antioxidant capacity of thyme oil emulsions determined with DPPH assay

Sample ID	Concentration	Emulsion type	Antioxidant Capacity (µmol Trolox/gr emulsion)
T2-P	2	Primary	0.84 ± 0.02^{a}
T2-H	2	Homogenized	0.84 ± 0.02^{a}
T4-P	4	Primary	0.90 ± 0.04^{a}
T4-H	4	Homogenized	0.88 ± 0.04^{a}

Table 3.5: Antioxidant capacity of clove oil emulsions determined with FRAP assay

Sample ID	Concentration	Emulsion type	Antioxidant Capacity (µmol Trolox/gr emulsion)
C2-P	2	Primary	42.58 ± 4.02^{c}
C2-H	2	Homogenized	39.60 ± 3.39^{c}
C4-P	4	Primary	116.72 ± 7.64^{a}
C4-H	4	Homogenized	85.99 ± 7.35^{b}

Table 3.6: Antioxidant capacity of thyme oil emulsions determined with FRAP assay

Sample ID	Concentration	Emulsion type	Antioxidant Capacity (µmol Trolox/gr emulsion)
T2-P	2	Primary	0.22 ± 0.02^{b}
T2-H	2	Homogenized	0.22 ± 0.01^{b}
T4-P	4	Primary	0.33 ± 0.03^{a}
T4-H	4	Homogenized	0.35 ± 0.03^{a}

3.4.2.2 Effect of Concentration on Antioxidant Capacity

During the experiments, essential oils were put into emulsions at two different concentrations (2% and 4%). The results can be seen in Table 3.3, Table 3.4, Table 3.5 and Table 3.6. Finally, it can be concluded that as concentration increased, antioxidant capacity increased in clove oil emulsions ($p<0.05$). This relation was valid for both DPPH and FRAP assays. The change was not an unexpected result as clove oil is known to have high antioxidant capacity which means even changes in small amounts could change the capacity (Gülçin, Elmastaş, & Aboul-Enein, 2012; Khaled F. M, Khaled M. A. Ramadan, & I. S. Ashoush., 2014). 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.

3.5 Total Phenolic Content with Folin-Ciocalteu (FC) Method

3.5.1 Total Phenolic Content of Pure Essential Oils

Total phenolic content of pure essential oils was measured, too. Results of the assay are 409.9 ± 26.8 mg GA/gr oil and 167.2 ± 2.8 mg GA/gr oil for clove oil and thyme oil, respectively. The findings were consistent with the previous studies. According to the study where different parts of clove were analyzed, total phenolic content (TPC) was in the range between 161.95-530.56 mg GA/g of extract (Ivanovic, Dimitrijevic-brankovic, Misic, & Ristic, 2012). In their study, they gave a range of 132 ± 4.4 and 334 ± 18.4 mg GA/g of extract for thyme oil obtained with different ways (Gallego, Gordon, Segovia, & Skowyra, 2013).

In conclusion, this study reveals that clove oil has more phenolic content and it shows more antioxidant capacity than thyme oil ($p<0.05$). In their research where different essential oils, including clove and thyme oil, were examined, Turgay & Esen (2015) found out that clove oil showed the highest antioxidant capacity together with the highest total phenolic content.

3.5.2 Effect of Microfluidization and Concentration on Total Phenolic Content of Emulsions

As it is clear in Table 3.7 and Table 3.8, thyme oil emulsion seems to be affected by the microfluidization. For clove oil emulsions, on the other hand, only 4% one was affected. C2-P kept its phenolic content ($p>0.05$).

The effect of concentration is common for all emulsions. As concentration increased, the total phenolic content increased in all samples ($p<0.05$). Results are in accordance with the study where phenolic content increased while concentration increased due to higher amount of bioactive compounds (Kalinowska, Gryko, Wróblewska, & Trypuć, 2020).

Table 3.7: Total phenolic content results of clove oil emulsions

Sample ID	Concentration	Emulsion type	Total phenolic content (mg GA/gr emulsion)
C2-P	2	Primary	6.79 ± 0.50^{c}
C2-H	2	Homogenized	6.12 ± 0.29^{c}
C4-P	4	Primary	19.36 ± 1.90^{a}
C4-H	4	Homogenized	15.26 ± 0.75^{b}

Table 3.8: Total phenolic content results of thyme oil emulsions

Sample ID	Concentration	Emulsion type	Total phenolic content (mg GA/gr emulsion)
T2-P	2	Primary	3.20 ± 0.20^{b}
T2-H	2	Homogenized	2.64 ± 0.13^{c}
T4-P	4	Primary	6.28 ± 0.47^{a}
T4-H	4	Homogenized	3.02 ± 0.16^{bc}

3.5.3 Correlation Between Folin-Ciocalteu Method and Antioxidant Capacity Methods

Phenolic content measurement is critical for antioxidants because this property is commonly associated with the phenolic structure (Moisa et al., 2018). Therefore, the correlation was checked between the total phenolic content and antioxidant capacity results. Pearson correlation values are given in Table 3.9. It is seen that there is a strong positive correlation between the FC and FRAP results for clove oil emulsions. The correlation between FC and DPPH is also similar. Although thyme oil correlations are not that high, there is still a positive correlation between FC and antioxidant capacity measured with FRAP method. However, no significant correlation was found between FC and DPPH results for thyme oil. Although there are some studies claiming the presence of a good correlation between phenolic

content and antioxidant capacity, there are many studies suggesting the opposite case. Findings of these studies are in accordance with the low correlation results (Kamiloğlu, Ercisli, Şengül, Toplu, & Serçe, 2009; Rafat, Philip, & Muniandy, 2010).

The good correlation for clove oil is important because it justifies the fact that phenolic structure provides the antioxidant property. In the case of thyme oil, it should be noted that there are some other compounds, such as flavonoids, that contribute to the antioxidant property. Actually, flavonoids are polyphenolic structure. So, they react with FC reagent, too. However, the misleading arises from the fact that FC method does not measure only phenolic substances but also reducing agents such as ascorbic acid, Maillard products, or some proteins and amino acids because they can interfere with FC reagent (Georgé, Brat, Alter, & Amiot, 2005). In fact, composition of thyme is very region dependent. It belongs to the huge Lamiaceae family whose members are spreading all over the world and the situation with thyme oil is not like eugenol making up the large percentage of clove oil. Therefore, the composition of thyme changes depending on where they grew, when they were harvested and the environmental conditions, etc. Therefore, sometimes FC method might not provide sensitivity for the detection of target compounds. Therefore, in order to have a distinction, the total flavonoid content measurement is also important.

There are also other points of view on the low correlation values between phenolic content and antioxidant capacity. Some studies claimed that the antioxidant capacity of essential oils might rely on the interaction between the components rather than the amount (Adaramola & Onigbinde, 2016; Mimica-Dukić, Orč Ić, Lesjak, & Šibul, 2016). These may explain the lower correlation between FC and antioxidant capacity results for thyme oil.

Table 3.9: Correlation constants between FC and antioxidant capacity methods

DPPH		FRAP	
Clove	**Thyme**	**Clove**	**Thyme**
0.963*	-	0.977*	0.486*

*All correlations were found to be statistically significant ($p<0.05$)

3.6 Stability

3.6.1 Droplet Size and PDI Values

Droplet size measurements were first done to freshly prepared emulsions. They were measured every 2 weeks for 1 month thereafter. The first day's results were compared in the previous sections. However, changes in the droplet size during the storage time is topic of stability. Droplet size and its change for all emulsion types are given in Table 3.10. According to the results, droplet size of clove oil emulsions increased within 15 days. On the other hand, thyme oil seems consistent within this one month period. The possible reason for instability will be discussed later.

Table 3.10: Droplet size results clove emulsions within one month

Sample ID	**Droplet Size (nm)**		
	1st day	**15th day**	**30th day**
C2-P	$72.85 \pm 0.84^{c,B}$	$189.03 \pm 15.66^{a,A}$	$180.57 \pm 1.21^{a,A}$
C2-H	$102.33 \pm 2.32^{b,B}$	$175.50 \pm 10.67^{a,A}$	$186.77 \pm 11.53^{a,A}$
C4-P	$102.55 \pm 5.04^{b,B}$	$183.50 \pm 3.88^{a,A}$	$175.37 \pm 2.32^{a,A}$
C4-H	$118.40 \pm 5.06^{a,B}$	$183.87 \pm 4.08^{a,A}$	$174.00 \pm 7.01^{a,A}$

Different small letters indicate significant differences among the samples in the same column; Capital letters indicate significant differences among the samples in the same row

Table 3.11: Droplet size results of thyme emulsions within one month

Sample ID	Droplet Size (nm)		
	1st day	**15th day**	**30th day**
T2-P	$179.37 \pm 13.19^{a,A}$	$176.67 \pm 12.38^{a,A}$	$164.40 \pm 11.5^{a,A}$
T2-H	$109.17 \pm 5.76^{b,A}$	$115.23 \pm 5.54^{b,A}$	$110.80 \pm 3.92^{b,A}$
T4-P	$177.35 \pm 5.74^{a,A}$	$172.10 \pm 5.11^{a,A}$	$181.60 \pm 5.39^{a,A}$
T4-H	$168.70 \pm 3.72^{a,A}$	$170.10 \pm 2.26^{a,A}$	$175.53 \pm 1.05^{a,A}$

Different small letters indicate significant differences among the samples in the same column; Capital letters indicate significant differences among the samples in the same row

PDI values of emulsions were also recorded each time the droplet size was measured. The first day results were given in Table 3.2 in the previous sections. PDI has been mostly reported to be related with the stability of the emulsion systems (Nagaraju et al., 2020). However, it is not correct to say that clove oil emulsions had good overall stability by only focusing on their relatively low PDI values on the first day. Instead, to observe PDI changes over time and make comments accordingly is much more proper. PDI values over one month are given in Table 3.12 and Table 3.13. According to the results, there is no change in PDI values for thyme oil emulsions. On the contrary, there is an increase in PDI values of clove oil. PDI changing within 15 days indicates destabilization started. PDI change can be the indicator of instability. However, PDI value is not controlling stability by itself only, but there are other factors, as well. Especially in essential oil emulsion systems, the solubility of the dispersed phase is the most critical parameter (Park et al., 2020).

Table 3.12: PDI values of homogenized clove oil over one month

Sample ID	1st day	15th day	30th day
C2-H	0.212 ± 0.018^{b}	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}

Different letters indicate significant differences in the same row; all emulsion types were analyzed individually

Table 3.13: PDI values of homogenized thyme oil over one month

Sample ID	1st day	15th day	30th day
T2-H	0.235 ± 0.001^{a}	0.260 ± 0.001^{a}	0.235 ± 0.001^{a}
T4-H	0.125 ± 0.006^{a}	0.114 ± 0.010^{a}	0.112 ± 0.002^{a}

Different letters indicate significant differences in the same row; all emulsion types were analyzed individually

3.6.2 Instantaneous 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.

3.6.3 Long Term Stability

For long term stability, firstly, a visual analysis was done. Homogenized clove oil emulsions were seemed to have sedimentation problem after 1 month. There was a distinct phase separation after 3 months. The appearance of homogenized thyme oil emulsions did not change during months. Therefore, they were kept centrifuged in specific periods. Appearance of emulsions after 3 months are given in Figure 3.4.

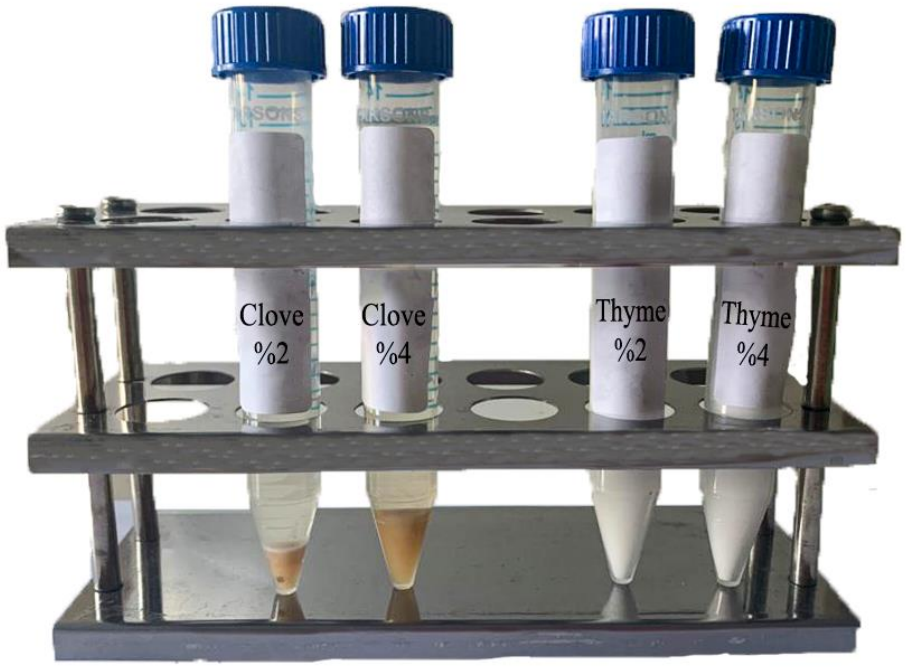


Figure 3.4: Appearance of emulsions after 3 months

Finally, it can be concluded that the emulsification technique and surfactant ratio were enough to obtain stable thyme oil emulsions. Constant droplet size and PDI values contribute to this result, too. However, the destabilization mechanism of clove oil emulsions should be made clear. As mentioned before, the smaller droplet size in clove oil emulsions was attributed to better adsorption of the surfactant.

Good adsorption actually brings smaller droplets but also less repulsion due to less bulky group which causes instability. To understand that, it is useful to clarify the difference between an emulsifier and a stabilizer. The term emulsifier is mostly used for the amphiphilic agents, which can adsorb on the interface and bring immiscible fluids together. Stabilizers are the agents that stabilize the emulsions for a certain period of time (Costa et al., 2019). Although they are used interchangeably sometimes, unstable emulsions remind the necessity of both actions. Adsorption theory offers that adsorption of the emulsifier is vital for stabilizing colloidal systems. However, good adsorption of emulsifiers to the

interface does not always mean that they are enough to keep the emulsions stable for a long time. Emulsifiers orient themselves in a way that the hydrophobic part lies towards oil and the hydrophilic part lies towards the water (Tadros, 2013). Better adsorption of nonionic surfactants means less amount of hydrophilic part in the continuous phase in O/W emulsions. This helps to create smaller droplets but causes less repulsion (Jemaa, Falleh, & Ksouri, 2019). Because fewer bulky groups of surfactants accumulate in the continuous phase, a weak stearic repulsion occurs. Reduced repulsion eventually causes emulsions to break down.

As mentioned earlier, emulsions break down with several mechanisms. They are more resistant against gravitational separations because Brownian motion dominates the gravitational forces. However, Ostwald ripening is inevitable (Mason et al., 2006). Ostwald ripening is a usual phenomenon in emulsions with essential oils because it is related to solubility. Considering that clove oil emulsion was unstable, it can be concluded that Ostwald ripening occurred, resulting in coalescence at the end. Authors compared the solubility of thymol, carvacrol, and eugenol found that eugenol had the highest solubility in water at room temperature (Chen, Michael Davidson, & Zhong, 2014). Another study demonstrated that eugenol had higher solubility in water than carvacrol (Ben Arfa, Combes, Preziosi-Belloy, Gontard, & Chalier, 2006). This information is 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 Ostwald ripening and coalescence. Besides, there are many studies observed instability in clove oil emulsions (Purwanti et al., 2018; M. Sharma et al., 2017).

3.7 TD-NMR Analysis

NMR measurement was applied to both pure essential oils and emulsions. Results of relaxation times for pure essential oils are consistent with the viscosity values.

There is an inverse relation between them, meaning that as viscosity increases, T_1 and T_2 values decrease (Heng Wang, Taborda, Alvarado, & Cortés, 2019). NMR and viscosity results of pure oils are given in Table 3.14 and Table 3.1, respectively. According to the tables, it is clear that thyme oil is more viscous than clove oil. This difference explains thyme oil had shorter relaxation times.

NMR results of emulsions are given in Table 3.15 and Table 3.16. It is seen that as the concentration of oil increases, T_1 relaxation time decreases in both thyme and clove oil emulsions. This is not an unexpected result because oils have shorter T_1 values than water (Hashemi et al., 2012).

When T_2 results were considered, a significant change was seen only emulsion with 2% thyme oil. Reminding the droplet size results of the first day, it makes sense. Because similar to NMR results, droplet size was decreasing after homogenizing for that sample only. Concluding homogenization was better, one can say that water became more restricted. Restriction of the water mobility causes water molecules to relax slower. Therefore, as the concentration of free water decreases in the environment, T_2 decreases (Rismanto & Zwaag, 2007).

Table 3.14: T_1 and T_2 results of pure oils

Clove oil		**Thyme oil**	
T_1 (ms)	**T_2 (ms)**	**T_1 (ms)**	**T_2 (ms)**
461.4 ± 24.8	405.5 ± 26.0	232.5 ± 14.5	199.3 ± 7.31

Table 3.15: T_1 and T_2 results of all clove oil emulsion types

Sample ID	T_1 (ms)	T_2 (ms)
C2-P	2127.0 ± 44.0^{ab}	1170.0 ± 35.0^{a}
C2-H	2320.0 ± 11.0^{a}	1142.5 ± 65.5^{a}
C4-P	2049.3 ± 41.1^{b}	1086.0 ± 62.0^{a}
C4-H	1968.5 ± 70.5^{b}	1152.0 ± 16.0^{a}

Table 3.16: T_1 and T_2 results of all thyme oil emulsion types

Sample ID	T_1 (ms)	T_2 (ms)
T2-P	2280.0 ± 88.2^{a}	1418.0 ± 21.6^{a}
T2-H	2241.0 ± 48.0^{a}	1288.0 ± 75.7^{b}
T4-P	1894.3 ± 47.8^{b}	1189.7 ± 100.2^{b}
T4-H	2039.7 ± 100.1^{ab}	1274.0 ± 12.4^{b}

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

This study aimed to obtain stable emulsions with essential oils so that they could be used in foods without any concerns about the amount of the essential oils. To fulfill this purpose, two different types of essential oils were used with two different concentrations. Clove oil and thyme oil were selected as essential oils due to their common use in Turkish cuisine. Besides, two different emulsions were formed, one using a high shear mixer and the other further homogenized with a microfluidizer.

Firstly, the droplet size of the emulsions was measured during one month of storage time. PDI and zeta potential values of the emulsions were also checked. It turned out that smaller droplets were formed with clove oil. It was attributed to the better interaction between surfactant and clove oil than that of thyme oil. However, an unusual result was obtained for the clove oil emulsions. An increase in droplet size after microfluidization was unexpected. The reason was thought to be the recoalescence of the droplets in the homogenization chamber. According to this phenomenon, it was concluded that an extense amount of energy was applied to the clove oil emulsions. Therefore, droplets accelerated unnecessarily and became larger by colliding with each other. Emulsions were observed with the Transmission Electron Microscopy as well. The results of TEM were confirmed with the droplet size results. Also, TEM confirmed that emulsions had spherical droplets.

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 a really long time. However, destabilization started in clove oil emulsions right

after homogenization. The mechanism of destabilization was thought to be as Ostwald ripening.

Essential oils are known to possess antioxidant property, and it mostly arises from their phenolic structures. Therefore, antioxidant capacity tests were applied to emulsions and pure essential oils both. Overall, emulsions still had remarkable antioxidant capacity after homogenization processes. Clove oil emulsions showed higher antioxidant capacity. After phenolic content measurements, a good correlation was found between the phenolic content and antioxidant capacity of clove oil, as was expected. In the case of thyme oil, a relatively lower correlation was found.

Finally, TD-NMR analysis was conducted. T_1 and T_2 relaxation times were measured for both emulsions and pure oils. NMR results of pure oils were explained with the viscosity values. Thyme oil was more viscous, and it gave lower T_1 and T_2 times. Changes of T_1 and T_2 times in emulsions, on the other hand, was explained with the mobility of water. In general, as oil concentration increased, relaxation times decreases due to less free mobile water in the environment.

To sum up, 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 selection is important. The solubility of essential oils is another thing that should be taken into account before emulsification. 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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APPENDICES

A. Calibration Curves

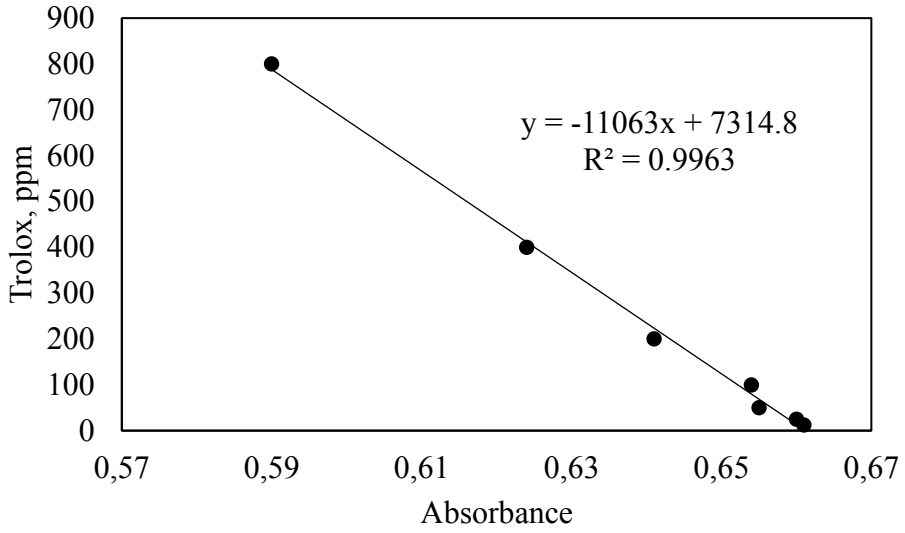

Figure A.1: Calibration curve for DPPH method

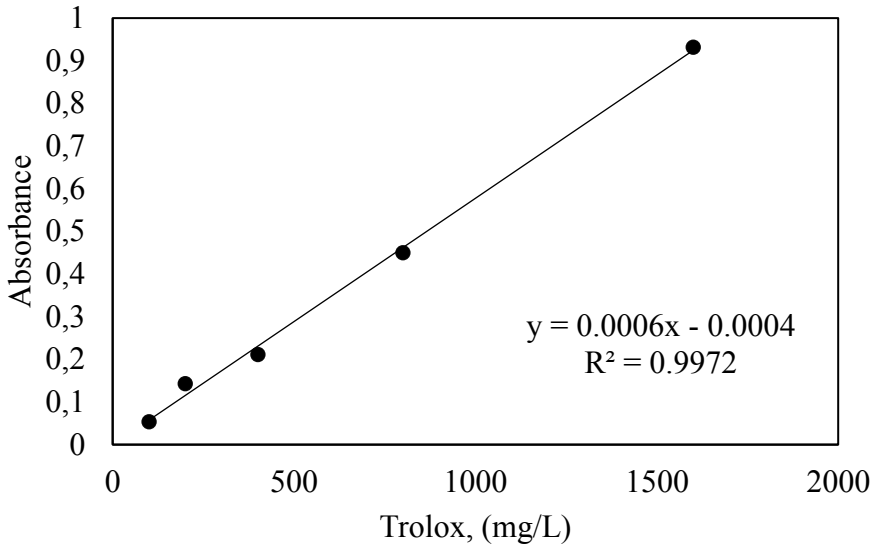

Figure A.2: Calibration curve for FRAP method

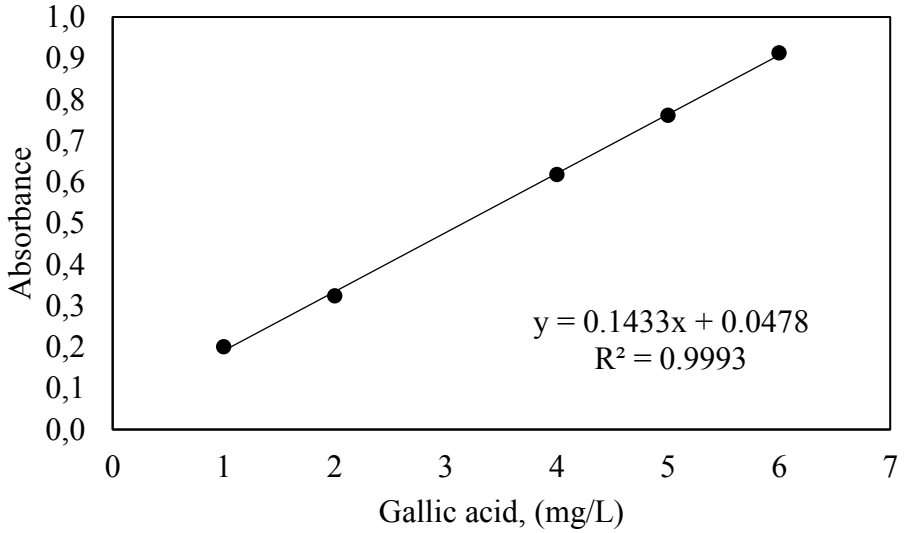

Figure A.3: Calibration curve for Folin-Ciocalteu method

B. Appendix Title

Table B.1: ANOVA for droplet size values of clove emulsions at 1st day

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
emulsion type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	1570,9	1570,94	71,87	0,000
emulsion type	1	1541,8	1541,79	70,54	0,000
concentration*emulsion type	1	139,4	139,40	6,38	0,036
Error	8	174,9	21,86		
Total	11	3427,0			

Table B.2: Comparison tests for droplet size values of clove emulsions at 1st day

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
4	6	110,473	A
2	6	87,590	B

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
homogenized	6	110,367	A
primary	6	87,697	B

Tukey Pairwise Comparisons: concentration*emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*emulsion type	N	Mean	Grouping
4 homogenized	3	118,400	A
4 primary	3	102,547	B
2 homogenized	3	102,333	B
2 primary	3	72,847	C

Table B.3: ANOVA for droplet size values of thyme emulsions at 1st day

Method

Factor coding (-1; 0; +1)
Rows unused 1

Factor Information

Factor	Type	Levels	Values
emulsion type	Fixed	2	homogenized; primary
concentration	Fixed	2	2; 4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
emulsion type	1	3730,4	3730,4	34,84	0,001
concentration	1	1984,9	1984,9	18,54	0,005
emulsion type*concentration	1	2273,0	2273,0	21,23	0,004
Error	6	642,4	107,1		
Total	9	10094,2			

Table B.4: Comparison tests for droplet size values of thyme emulsions at 1st day

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
primary	5	178,358	A
homogenized	5	138,933	B

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
4	4	173,025	A
2	6	144,267	B

Tukey Pairwise Comparisons: emulsion type*concentration

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type*concentration	N	Mean	Grouping
primary 2	3	179,367	A
primary 4	2	177,350	A
homogenized 4	2	168,700	A
homogenized 2	3	109,167	B

Table B.5: ANOVA for droplet size values of emulsions at 15th day

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
essential oil	Fixed	2	0; 1
concentration	Fixed	2	2; 4
type	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
essential oil	1	3586,8	3586,8	31,56	0,000
concentration	1	1058,7	1058,7	9,32	0,008
type	1	2200,3	2200,3	19,36	0,000
essential oil*concentration	1	844,9	844,9	7,43	0,015
essential oil*type	1	947,5	947,5	8,34	0,011
concentration*type	1	2016,7	2016,7	17,75	0,001
essential oil*concentration*type	1	777,5	777,5	6,84	0,019
Error	16	1818,3	113,6		
Total	23	13250,7			

Table B.6: Comparison tests for droplet size values of emulsions at 15th day

Tukey Pairwise Comparisons: essential oil

Grouping Information Using the Tukey Method and 95% Confidence

essential oil	N	Mean	Grouping
0	12	182,975	A
1	12	158,525	B

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
4	12	177,392	A
2	12	164,108	B

Tukey Pairwise Comparisons: type

Grouping Information Using the Tukey Method and 95% Confidence

type	N	Mean	Grouping
0	12	180,325	A
1	12	161,175	B

Tukey Pairwise Comparisons: essential oil*concentration

Grouping Information Using the Tukey Method and 95% Confidence

essential oil*concentration	N	Mean	Grouping
0 4	6	183,683	A
0 2	6	182,267	A
1 4	6	171,100	A
1 2	6	145,950	B

Tukey Pairwise Comparisons: essential oil*type

Grouping Information Using the Tukey Method and 95% Confidence

essential oil*type	N	Mean	Grouping
0 0	6	186,267	A
0 1	6	179,683	A
1 0	6	174,383	A
1 1	6	142,667	B

Tukey Pairwise Comparisons: concentration*type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*type	N	Mean	Grouping
2 0	6	182,850	A
4 0	6	177,800	A
4 1	6	176,983	A
2 1	6	145,367	B

Tukey Pairwise Comparisons: essential oil*concentration*type

Grouping Information Using the Tukey Method and 95% Confidence

essential oil*concentration*type	N	Mean	Grouping
0 2 0	3	189,033	A
0 4 1	3	183,867	A
0 4 0	3	183,500	A
1 2 0	3	176,667	A
0 2 1	3	175,500	A
1 4 0	3	172,100	A
1 4 1	3	170,100	A
1 2 1	3	115,233	B

Table B.7: ANOVA for droplet size values of emulsions at 30th day

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
essential oil	Fixed	2	0; 1
concentration	Fixed	2	2; 4
type	Fixed	2	0; 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
essential oil	1	2512,1	2512,14	38,94	0,000
concentration	1	1444,1	1444,14	22,38	0,000
type	1	1061,2	1061,19	16,45	0,001
essential oil*concentration	1	3522,4	3522,36	54,59	0,000
essential oil*type	1	1468,3	1468,32	22,76	0,000
concentration*type	1	563,8	563,77	8,74	0,010
essential oil*concentration*type	1	1071,5	1071,53	16,61	0,001
Error	15	967,8	64,52		
Total	22	13320,3			

Table B.8: Comparison tests for droplet size values of emulsions at 30th day

Tukey Pairwise Comparisons: essential oil

Grouping Information Using the Tukey Method and 95% Confidence

essential oil	N	Mean	Grouping
0	12	179,175	A
1	11	158,083	B

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
4	12	176,625	A
2	11	160,633	B

Tukey Pairwise Comparisons: type

Grouping Information Using the Tukey Method and 95% Confidence

type	N	Mean	Grouping
0	11	175,483	A

1	12	161,775	B

Tukey Pairwise Comparisons: essential oil*concentration

Grouping Information Using the Tukey Method and 95% Confidence

essential oil*concentration	N	Mean	Grouping
0 2	6	183,667	A
1 4	6	178,567	A
0 4	6	174,683	A
1 2	5	137,600	B

Tukey Pairwise Comparisons: essential oil*type

Grouping Information Using the Tukey Method and 95% Confidence

essential oil*type	N	Mean	Grouping
0 1	6	180,383	A
0 0	6	177,967	A
1 0	5	173,000	A
1 1	6	143,167	B

Tukey Pairwise Comparisons: concentration*type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*type	N	Mean	Grouping
4 0	6	178,483	A
4 1	6	174,767	A
2 0	5	172,483	A
2 1	6	148,783	B

Tukey Pairwise Comparisons: essential oil*concentration*type

Grouping Information Using the Tukey Method and 95% Confidence

essential oil*concentration*type	N	Mean	Grouping
0 2 1	3	186,767	A
1 4 0	3	181,600	A
0 2 0	3	180,567	A
1 4 1	3	175,533	A
0 4 0	3	175,367	A
0 4 1	3	174,000	A
1 2 0	2	164,400	A
1 2 1	3	110,800	B

Table B.9: ANOVA for changes in the droplet size within the time for primary emulsions with 2% - clove oil

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
time (day)	Fixed	3	0; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
time (day)	2	25174,6	12587,3	101,78	0,000
Error	6	742,0	123,7		
Total	8	25916,6			

Table B.10: Comparison tests for changes in the droplet size within the time for primary emulsions with 2% - clove oil

Tukey Pairwise Comparisons: time (day)

Grouping Information Using the Tukey Method and 95% Confidence

time (day)	N	Mean	Grouping
15	3	189,033	A
30	3	180,567	A
0	3	72,847	B

Table B.11: ANOVA for changes in the droplet size within the time for homogenized emulsions with 2% - clove oil

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
time (day)	Fixed	3	0; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
time (day)	2	12609,3	6304,6	50,00	0,000
Error	6	756,6	126,1		
Total	8	13365,9			

Table B.12: Comparison tests for changes in the droplet size within the time for homogenized emulsions with 2% - clove oil

Tukey Pairwise Comparisons: time (day)

Grouping Information Using the Tukey Method and 95% Confidence

time (day)	N	Mean	Grouping
30	3	186,767	A
15	3	175,500	A
0	3	102,333	B

Table B.13: ANOVA for changes in the droplet size within the time for primary emulsions with 4% - clove oil

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
time (day)	Fixed	3	0; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
time (day)	2	11922,3	5961,17	253,60	0,000
Error	6	141,0	23,51		
Total	8	12063,4			

Table B.14: Comparison tests for changes in the droplet size within the time for primary emulsions with 4% - clove oil

Tukey Pairwise Comparisons: time (day)

Grouping Information Using the Tukey Method and 95% Confidence

time (day)	N	Mean	Grouping
15	3	183,500	A
30	3	175,367	A
0	3	102,547	B

Table B.15: ANOVA for changes in the droplet size within the time for homogenized emulsions with 4% - clove oil

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
time (day)	Fixed	3	0; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
time (day)	2	7474,6	3737,30	81,79	0,000
Error	6	274,2	45,69		
Total	8	7748,8			

Table B.16: Comparison tests for changes in the droplet size within the time for homogenized emulsions with 4% - clove oil

Tukey Pairwise Comparisons: time (day)

Grouping Information Using the Tukey Method and 95% Confidence

time (day)	N	Mean	Grouping
15	3	183,867	A
30	3	174,000	A
0	3	118,400	B

Table B.17: ANOVA for changes in the droplet size within the time for primary emulsions with 2% - thyme oil

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
time (day)	Fixed	3	0; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
time (day)	2	289,1	144,5	0,58	0,594
Error	5	1246,2	249,2		
Total	7	1535,2			

Table B.18: Comparison tests for changes in the droplet size within the time for primary emulsions with 2% - thyme oil

Tukey Pairwise Comparisons: time (day)

Grouping Information Using the Tukey Method and 95% Confidence

time (day)	N	Mean	Grouping
0	3	179,367	A
15	3	176,667	A
30	2	164,400	A

Table B.19: ANOVA for changes in the droplet size within the time for homogenized emulsions with 2% - thyme oil

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
time (day)	Fixed	3	0; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
time (day)	2	59,13	29,56	0,75	0,513
Error	6	237,65	39,61		
Total	8	296,78			

Table B.20: Comparison tests for changes in the droplet size within the time for homogenized emulsions with 2% - thyme oil

Tukey Pairwise Comparisons: time (day)

Grouping Information Using the Tukey Method and 95% Confidence

time (day)	N	Mean	Grouping
15	3	115,233	A
30	3	110,800	A
0	3	109,167	A

Table B.21: ANOVA for changes in the droplet size within the time for primary emulsions with 4% - thyme oil

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
time (day)	Fixed	3	0; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
time (day)	2	156,1	78,03	1,77	0,249
Error	6	264,7	44,12		
Total	8	420,8			

Table B.22: Comparison tests for changes in the droplet size within the time for primary emulsions with 4% - thyme oil

Tukey Pairwise Comparisons: time (day)

Grouping Information Using the Tukey Method and 95% Confidence

time (day)	N	Mean	Grouping
30	3	181,600	A
0	3	173,633	A
15	3	172,100	A

Table B.23: ANOVA for changes in the droplet size within the time for homogenized emulsions with 4% - thyme oil

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
time (day)	Fixed	3	0; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
time (day)	2	49,73	24,86	2,47	0,165
Error	6	60,33	10,06		
Total	8	110,06			

Table B.24: Comparison tests for changes in the droplet size within the time for homogenized emulsions with 4% - thyme oil

Tukey Pairwise Comparisons: time (day)

Grouping Information Using the Tukey Method and 95% Confidence

time (day)	N	Mean	Grouping
30	3	175,533	A
0	3	171,167	A
15	3	170,100	A

Table B.25: ANOVA for PDI values of clove oil emulsions on the first day

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
emulsion type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	0,048110	0,048110	199,47	0,000
emulsion type	1	0,029260	0,029260	121,31	0,000
concentration*emulsion type	1	0,018550	0,018550	76,91	0,000
Error	6	0,001447	0,000241		
Total	9	0,109068			

Table B.26: Comparisons for PDI values of clove oil emulsions on the first day

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
2	6	0,310833	A
4	4	0,169250	B

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
primary	5	0,295250	A
homogenized	5	0,184833	B

Tukey Pairwise Comparisons: concentration*emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*emulsion type	N	Mean	Grouping
2 primary	3	0,410000	A
2 homogenized	3	0,211667	B
4 primary	2	0,180500	B C
4 homogenized	2	0,158000	C

Table B.27: ANOVA for PDI values of thyme oil emulsions on the first day

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
emulsion type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	0,000317	0,000317	2,34	0,177
emulsion type	1	0,144649	0,144649	1065,77	0,000
concentration*emulsion type	1	0,021812	0,021812	160,71	0,000
Error	6	0,000814	0,000136		
Total	9	0,170810			

Table B.28: Comparison tests for PDI values of thyme oil emulsions on the first day

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
2	5	0,310417	A
4	5	0,298917	A

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
primary	5	0,427417	A
homogenized	5	0,181917	B

Tukey Pairwise Comparisons: concentration*emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*emulsion type	N	Mean	Grouping
4 primary	3	0,469333	A
2 primary	2	0,385500	B
2 homogenized	3	0,235333	C
4 homogenized	2	0,128500	D

Table B.29: ANOVA for PDI values of emulsion with 2% clove oil over one month

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
day	Fixed	3	1; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
day	2	0,014601	0,007301	11,78	0,013
Error	5	0,003099	0,000620		
Total	7	0,017700			

Table B.30: Comparisons for PDI values of emulsion with 2% clove oil over one month

Tukey Pairwise Comparisons: day

Grouping Information Using the Tukey Method and 95% Confidence

day	N	Mean	Grouping
30	2	0,317000	A
15	3	0,280000	A
1	3	0,211667	B

Table B.31: ANOVA for PDI values of emulsion with 4% clove oil over one month

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
Day	Fixed	3	1; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Day	2	0,019666	0,009833	33,13	0,003
Error	4	0,001187	0,000297		
Total	6	0,020853			

Table B.32: Comparisons for PDI values of emulsion with 4% clove oil over one month

Tukey Pairwise Comparisons: Day

Grouping Information Using the Tukey Method and 95% Confidence

Day	N	Mean	Grouping
15	2	0,315500	A

30	3	0,284333	A	
1	2	0,183000		B

Table B.33: ANOVA for PDI values of emulsion with 2% thyme oil over one month

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
Day	Fixed	3	1; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Day	2	0,000865	0,000432	4,33	0,100
Error	4	0,000399	0,000100		
Total	6	0,001264			

Table B.34: Comparisons for PDI values of emulsion with 2% thyme oil over one month

Tukey Pairwise Comparisons: Day

Grouping Information Using the Tukey Method and 95% Confidence

Day	N	Mean	Grouping
15	2	0,260000	A
30	2	0,235500	A
1	3	0,235333	A

Table B.35: ANOVA for PDI values of emulsion with 4% thyme oil over one month

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
Day	Fixed	3	1; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Day	2	0,000324	0,000162	1,81	0,305
Error	3	0,000269	0,000090		
Total	5	0,000593			

Table B.36: Comparisons for PDI values of emulsion with 4% thyme oil over one month

Tukey Pairwise Comparisons: Day

Grouping Information Using the Tukey Method and 95% Confidence

Day	N	Mean	Grouping
1	2	0,1285	A
30	2	0,1140	A
15	2	0,1120	A

Table B.37: ANOVA for antioxidant capacity values of clove oil emulsions determined with DPPH method

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
emulsion type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	0,186674	0,186674	295380,51	0,000
emulsion type	1	0,000002	0,000002	2,39	0,142
concentration*emulsion type	1	0,000000	0,000000	0,13	0,719
Error	16	0,000010	0,000001		
Total	19	0,186686			

Table B.38: Comparison tests for antioxidant capacity values of clove oil emulsions determined with DPPH method

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping	
2	12	0,288646	A	
4	8	0,091440		B

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping

homogenized	10	0,190323	A
primary	10	0,189763	A

Tukey Pairwise Comparisons: concentration*emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*emulsion type	N	Mean	Grouping	
2 homogenized	6	0,288860	A	
2 primary	6	0,288432	A	
4 homogenized	4	0,091787		B
4 primary	4	0,091093		B

Table B.39: ANOVA for antioxidant capacity values of thyme oil emulsions with determined DPPH method

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
emulsion type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	0,008563	0,008563	5,18	0,044
emulsion type	1	0,000115	0,000115	0,07	0,797
concentration*emulsion type	1	0,000517	0,000517	0,31	0,587
Error	11	0,018191	0,001654		
Total	14	0,029014			

Table B.40: Comparison test for antioxidant capacity values of thyme oil emulsions determined with DPPH method

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping	
4	9	0,894264	A	
2	6	0,844289		B

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
primary	9	0,872170	A
homogenized	6	0,866383	A

Tukey Pairwise Comparisons: concentration*emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*emulsion type	N	Mean	Grouping
4 primary	6	0,903296	A
4 homogenized	3	0,885231	A
2 homogenized	3	0,847534	A
2 primary	3	0,841044	A

Table B.41: ANOVA for antioxidant capacity values of clove oil emulsions with determined FRAP method

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
emulsion type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	21507,2	21507,2	539,02	0,000
emulsion type	1	1675,0	1675,0	41,98	0,000
concentration*emulsion type	1	1144,2	1144,2	28,68	0,000
Error	20	798,0	39,9		
Total	23	26162,8			

Table B.42: Comparison tests for antioxidant capacity values of clove oil emulsions determined with FRAP method

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
4	11	101,355	A
2	13	41,058	B

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
primary	12	79,6203	A
homogenized	12	62,7932	B

Tukey Pairwise Comparisons: concentration*emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*emulsion type	N	Mean	Grouping		
4 primary	6	116,723	A		
4 homogenized	5	85,988		B	
2 primary	6	42,518			C
2 homogenized	7	39,598			C

Table B.43: ANOVA for antioxidant capacity values of thyme oil emulsions determined with FRAP method

Method

Factor coding (-1; 0; +1)

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
emulsion type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	0,070838	0,070838	116,72	0,000
emulsion type	1	0,000158	0,000158	0,26	0,616
concentration*emulsion type	1	0,000274	0,000274	0,45	0,511
Error	16	0,009710	0,000607		
Total	19	0,081734			

Table B.44: Comparison tests for antioxidant capacity values of thyme oil emulsions determined with FRAP method

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
4	8	0,339339	A
2	12	0,215451	B

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
homogenized	9	0,280324	A
primary	11	0,274466	A

Tukey Pairwise Comparisons: concentration*emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*emulsion type	N	Mean	Grouping
4 homogenized	3	0,346123	A
4 primary	5	0,332556	A
2 primary	6	0,216376	B
2 homogenized	6	0,214526	B

Table B.45: Correlation between DPPH and FRAP results for clove oil emulsions

Method

Correlation type	Pearson
Rows used	20

Correlations

	DPPH
FRAP	0,950

Table B.46: Correlation between DPPH and FRAP results for clove oil emulsions

Method

Correlation type	Pearson
Rows used	13

Correlations

	DPPH
FRAP	0,426

Table B.47: ANOVA for total phenolic content values of clove oil emulsions

Method

Factor coding (-1; 0; +1)
Rows unused 1

Factor Information

Factor	Type	Levels	Values
emulsion type	Fixed	2	homogenized; primary
concentration	Fixed	2	2; 4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
emulsion type	1	20,286	20,286	34,44	0,000
concentration	1	421,824	421,824	716,21	0,000
emulsion type*concentration	1	10,541	10,541	17,90	0,001
Error	11	6,479	0,589		
Total	14	450,752			

Table B.48: Comparison tests for total phenolic content values of clove oil emulsions

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
primary	7	13,0725	A
homogenized	8	10,6927	B

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
4	9	17,3084	A
2	6	6,4568	B

Tukey Pairwise Comparisons: emulsion type*concentration

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type*concentration	N	Mean	Grouping
primary 4	4	19,3560	A
homogenized 4	5	15,2609	B
primary 2	3	6,7889	C
homogenized 2	3	6,1246	C

Table B.49: ANOVA for total phenolic content values of thyme oil emulsions

Method

Factor coding (-1; 0; +1)
Rows unused 1

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
emulsion type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	11,4693	11,4693	286,52	0,000
emulsion type	1	13,8249	13,8249	345,37	0,000
concentration*emulsion type	1	7,1114	7,1114	177,65	0,000
Error	13	0,5204	0,0400		
Total	16	23,5307			

Table B.50: Comparison tests for total phenolic content values of tyhme oil emulsions

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
4	5	4,74845	A
2	12	2,91946	B

Tukey Pairwise Comparisons: emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

emulsion type	N	Mean	Grouping
primary	8	4,83798	A
homogenized	9	2,82993	B

Tukey Pairwise Comparisons: concentration*emulsion type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*emulsion type	N	Mean	Grouping		
4 primary	2	6,47258	A		
2 primary	6	3,20339		B	
4 homogenized	3	3,02433		B	C
2 homogenized	6	2,63553			C

Table B.51: ANOVA for T1 values of clove oil emulsion types

Method

Factor coding (-1; 0; +1)
Rows unused 3

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	100464	100464	26,27	0,004
type	1	6863	6863	1,79	0,238
concentration*type	1	40901	40901	10,70	0,022
Error	5	19121	3824		
Total	8	158972			

Table B.52: Comparison tests for T1 values of clove oil emulsion types

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
2	4	2223,50	A
4	5	2008,92	B

Tukey Pairwise Comparisons: type

Grouping Information Using the Tukey Method and 95% Confidence

type	N	Mean	Grouping
homogenized	4	2144,25	A
primary	5	2088,17	A

Tukey Pairwise Comparisons: concentration*type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*type	N	Mean	Grouping	
2 homogenized	2	2320,00	A	
2 primary	2	2127,00	A	B
4 primary	3	2049,33		B
4 homogenized	2	1968,50		B

Table B.53: ANOVA for T2 values of clove oil emulsion types

Method

Factor coding (-1; 0; +1)
Rows unused 4

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	2775,1	2775,1	0,58	0,490
type	1	741,1	741,1	0,15	0,715
concentration*type	1	4371,1	4371,1	0,91	0,394
Error	4	19230,5	4807,6		
Total	7	27117,9			

Table B.54: Comparison tests for T2 values of clove oil emulsion types

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping
2	4	1156,25	A
4	4	1119,00	A

Tukey Pairwise Comparisons: type

Grouping Information Using the Tukey Method and 95% Confidence

type	N	Mean	Grouping
homogenized	4	1147,25	A
primary	4	1128,00	A

Tukey Pairwise Comparisons: concentration*type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*type	N	Mean	Grouping
2 primary	2	1170,0	A
4 homogenized	2	1152,0	A
2 homogenized	2	1142,5	A
4 primary	2	1086,0	A

Table B.55: ANOVA for T1 values of thyme oil emulsion types

Method

Factor coding (-1; 0; +1)
Rows unused 1

Factor Information

Factor	Type	Levels	Values
concentration	Fixed	2	2; 4
type	Fixed	2	homogenized; primary

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
concentration	1	229713	229713	24,79	0,002
type	1	7538	7538	0,81	0,397
concentration*type	1	22653	22653	2,44	0,162
Error	7	64873	9268		
Total	10	339600			

Table B.56: Comparison tests for T1 values of thyme oil emulsion types

Tukey Pairwise Comparisons: concentration

Grouping Information Using the Tukey Method and 95% Confidence

concentration	N	Mean	Grouping	
2	5	2260,5	A	
4	6	1967,0		B

Tukey Pairwise Comparisons: type

Grouping Information Using the Tukey Method and 95% Confidence

type	N	Mean	Grouping
homogenized	5	2140,33	A
primary	6	2087,17	A

Tukey Pairwise Comparisons: concentration*type

Grouping Information Using the Tukey Method and 95% Confidence

concentration*type	N	Mean	Grouping	
2 primary	3	2280,00	A	
2 homogenized	2	2241,00	A	
4 homogenized	3	2039,67	A	B
4 primary	3	1894,33		B