FORMULATION AND CHARACTERIZATION OF CHIA SEED OIL NANOEMULSIONS

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

FORMULATION AND CHARACTERIZATION OF CHIA SEED OIL NANOEMULSIONS

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Chia seed is a good source of not only protein, dietary fiber and antioxidants but also polyunsaturated fatty acids, which are in higher amounts compared to other seed-based food sources. Nanoemulsions are colloidal dispersions that contain small particles, typically around 20-200 nm in diameter, that may not be distinctively visible since they do not scatter light strongly. Moreover, they have strong stability and can protect the embedded oil from oxidation. Thus, utilization of nanoemulsions from chia seed oil arouses interest among researchers. In this study, oil was extracted from grinded chia seed using conventional extraction methods and high pressure assisted extraction. In addition to oil extraction, chia oil nanoemulsions were prepared using spontaneous emulsification (0.5, 1, 1.25, 1.5, 2.5 and 3 % (w/w)). For chia oil emulsions, particle size analysis, antioxidant activity by DPPH and FRAP, total phenolic content by Folin Ciocalteu, peroxide value, determination of secondary oxidation products by TBARS, oil-water interaction by NMR Relaxometry, instantaneous and long term stability experiments were conducted. Also, HHP was applied to emulsion in order to see the coalescence mechanism. According to results obtained, emulsion systems showed better antioxidant activity and phenolic content than oil samples. In addition to this, among the emulsions, the best antioxidant activity, phenolic content and oxidative stability was found in the emulsion having 2.5 % (w)

chia oil. This study showed that formation of nanoemulsions helped to improve the bioavailability and bioactivity.

Keywords: Chia Oil, Spontaneous Emulsification, HHP, Antioxidant, Nanoemulsion, Low Energy Emulsification Method, Emulsion Stability

ÖZ

ÇİYA TOHUMLARINDAN YAĞ EKSTRAKSİYONU VE NANOEMÜLSİYON YAPIMI

Kaya, Eda Ceren
Yüksek Lisans, Gıda Mühendisliği
Tez Danışmanı: Prof. Dr. Hami Alpas

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Çiya tohumu, tarihi çok eski tarihsel dönemlerden beri bilinse de gıda ürünü olarak son zamanlarda ilgi uyandırmaya başlamıştır. Antioksidan ve omega 3,6 yağ asitleri açısından oldukça zengin olan çiya yağı, birçok tohum bazlı bitkisel kaynaklardan daha zengin besin değerlerine sahiptir. Çalışmada çiya tohumundan yağ ekstraksiyonu ve emülsiyon yapımı gerçekleştirilmiştir. Nanoemülsiyonlar parçacık boyutları oldukça küçük ve stabil kolloidal yapılardır. Aynı zamanda, stabiliteleri oldukça yüksek olduğundan, yağı okside olmadan koruyabilmektedir. Bu çalışmada, geleneksel ve yüksek hidrostatik basınç destekli yöntemlerle çiya tohumundan yağ ekstraksiyonu yapılmıştır. Bunun yanı sıra, kendiliğinden emülsiyon yöntemi kullanılarak çiya yağından nanoemülsiyon elde edilmiştir (0.5, 1, 1.25, 1.5, 2.5 and 3 % (w/w)). Nanoemülsiyonların antioksidan özelliği, parçacık boyutu ve oksidatif stabilitesi ve fenolik içeriği analiz edilmiştir. Çalışma içerisinde ayrıca yüksek hidrostatik basıncın emülsiyon stabilitesine etkisi de incelenmiştir. Sonuçlara göre, emülsiyonların aktif bileşenlerin etkisini arttırdığı gözlemlenmiştir. Ayrıca ağırlıkça 2.5% konsantrasyonlu emülsiyonun oksidatif stabilite, antioksidan, fenolik içerik açısından en ideal emülsiyon olduğu saptanmıştır

Anahtar Kelimeler: Çiya Tohumu, Çiya Yağı, Doymamış Yağlar, Antioksidan, Yüksek Hidrostatik Basınç, Kendiliğinden Emülsiyon Hazırlama, Emülsiyon Stabilitesi

To the one that deserve best, my beloved family…

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INTRODUCTION

1.1. General Information

1.1.1. Seed Oils

Due to the presence of phenolic compounds, natural antioxidants and other complex compounds, seed oils can be vital for the human diet (Wanasundara et al., 1997). Seed oil fatty acids which are mainly unsaturated, show chemical diversity in the form of triglycerides (95-98%), sterols, diglycerides, squalene, triterpenic dialcohols (Kongbonga.; Ghalila; Onana; Majdi.; Lakhdar; Mezlini & Ghalila, 2017). Besides this, fatty acids are preferred to be used in pharmaceutical and medical fields (Pandharinath, 2015; Warra et al., 2012). From well-known seed sources to very rare ones, seed oils could be obtained from various plant-based sources. Peanut, canola, palm kernel, cottonseed, macadamia, cashew, hazelnut can be given as examples (Chang, 2013).

1.1.2. Oil Seed Demand and Production

According to the survey data obtained from organizations, researchers and expert witnesses, food security concerns related to increasing world food demand is going to accelerate within thirty years (Food and Agriculture Organization of the United Nations, 2018). In order to meet the consumer's nutritional needs, seeds and oil from different sources could be very effective solutions due to their nutritional value, resistance to harsh environmental conditions and diversity. It is predicted that the use of seed and their oil derivatives are expected to increase within the next ten years

(Ouilly et al., 2017). Despite the difference in cultivation conditions, warm climate is generally needed for seed oil production. When the global production is examined, it is seen that, majority of seed oil is obtained in American and European continents (Sharma et al., 2012). However, due to drastic increase in population and environmental changes caused by global warming, advanced procurement strategies, different seed sources and oil products needs to be explored. Therefore, considering the current needs and trends, chia (*Salvia hispanica* L.) is thought to be a good alternative (Jat, Singh, Sharma, Rai, 2018).

1.1.3. Chia Seed

Chia, which also known as *Salvia hispanica* L., is the plant from the subgroup of *Lamiaceae* or *Labitae*. In ancient Central American civilizations, chia has been utilized as the dominant food source. Owing to its abundance and environmental adaptation, chia is prefered over traditional sources of edible legumes. In Tenochtitlan, which was the capital town of Aztec Empire, chia seeds were known as the booty of wars. Nearly 5-15,000 tons of seeds were accepted as prize each year. In addition to this, chia seed had been considered as 'holy' for 500 years. In ancient times, different uncontrolled and untamed species of chia seed were also grown. Today, only domestic type of *Salvia hispanica* is cultivated.

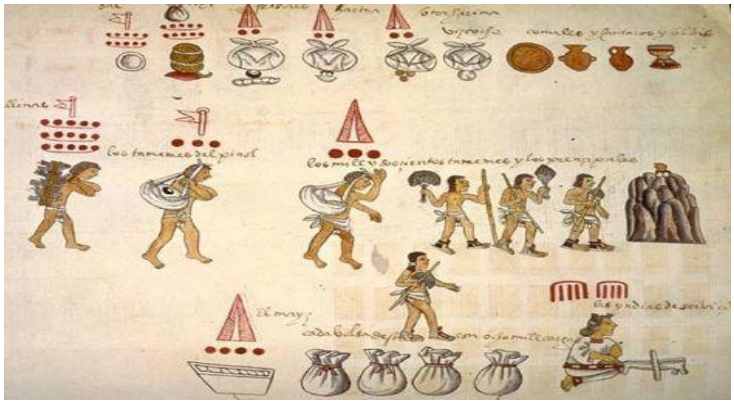

Figure 1.1. An Aztec Mural Representing Chia Seed Harvesting And Transportation (2018).

Chia seed includes high amount of nutritive components. Domestic chia seed is comprised of 15 - 25% protein, 30 - 33% fats, 26 - 41% carbohydrates, 18 - 30% dietary fiber, 4 - 5% ash, 90 - 93% dry matter, vitamins and minerals (Sharma et al., 2012). Most importantly, it is also composed of high amount of antioxidants (Ixtaina, Nolasco, and Tomas, 2008). Although chia seed and its oil products are commonly used in some regions of the world, it is not very familiar in European countries and Middle East. Therefore, to habituate the nations with this food source, some European governments are trying to work on different caseworks to inform chia seed to public. To set an example, some state authorities, such as Germany have accepted chia as a novel food and been the pioneer for its utilisation in bread by 5% (Commission of the European Communities, 2009).

Due to convenience of physical cultivation conditions, chia seeds are generally found in mild temperature zone. In these regions, chia is known to have future potential (Guiotto, Ixtaina, Tomás and Nolasco, 2013). Chia seeds can be divided into two main subgroups, namely black and white. Majority of chia seeds produced are of black type.

1.1.4. Chia Oil

Not only the rough seed portion, but specifically, oil of chia seeds has significant nutrient potential. Oil contains enormous amount of triglycerides of PUFA acids. Moreover, oleic acid constitutes the majority of polyunsaturated fatty acids. In addition to this, as a saturated fatty acid, palmitic acid is found in significant amount (Yakindra, Vongsvivut, Adhikari and Adhikari, 2017). Based on the cultivated area, the composition of omega 3 and 6 fatty acid groups may show diversity. For example, chia oil obtained from the seeds grown in Italy, Argentina, Mexico and United States were found to include omega 3 and 6 fatty acids by 62 % and 31 %. respectively (Bodoira, Penci, Ribotta and Martínez, 2017). As it has been known for years, PUFA groups are very beneficial due to their ability to reduce some diseases like heart related disorders, cholesterol and hypertension. Furthermore, chia oil could be more efficient compared to other PUFA rich sources since it is less susceptible to spoilage and side effects (Souza, Francisco, Sanchez, Guimarães-Inácio, Valderrama, E. Bona, Tanamati, Leimann and Gonçalves, 2017). On the other side, since chia oil contains significant amount of unsaturated fatty acid building blocks, it is important to protect chia oil from oxidation accelerating environmental conditions and to store it in proper conditions (Bodoira, Penci, Ribotta and Martínez, 2017).

There have been very few studies that focus on the chemical and physical properties and utilization of chia seed oil in literature. Thus, in this study the focus will be mostly on production and characterization of chia seed oil.

1.1.5. Market Position of Chia Seed and Oil

Drastic increase of human population and high consumption rate of food sources could force people to adopt different and more nutritional sources to meet the metabolic needs of human body. Because of this, chia seed and extracted oil have become popular in market shelves. European countries have imported and exported chia seeds in very high amount. According to the reported results (2019), Europe has managed to export more than thousands of tons of chia seeds, which have been revenued in high amount in the past couple of years (Global Chia Seed Market- Size, Share and Forecast, 2019). When supply and demand for chia seed and oil product are taken into consideration, it might be predicted that the chia seed market will expand in the following years.

When the Ministry of Foreign Affairs (2017) report was investigated, it was seen that important European countries had the biggest margin of profit in terms of importation up to this time. Usage of chia seed and its oil in pharmaceutical and cosmetic industry as well as food market shelves may make a great contribution to growth (Global Chia Seed Market- Size, Share and Forecast (2018-2025), 2019).

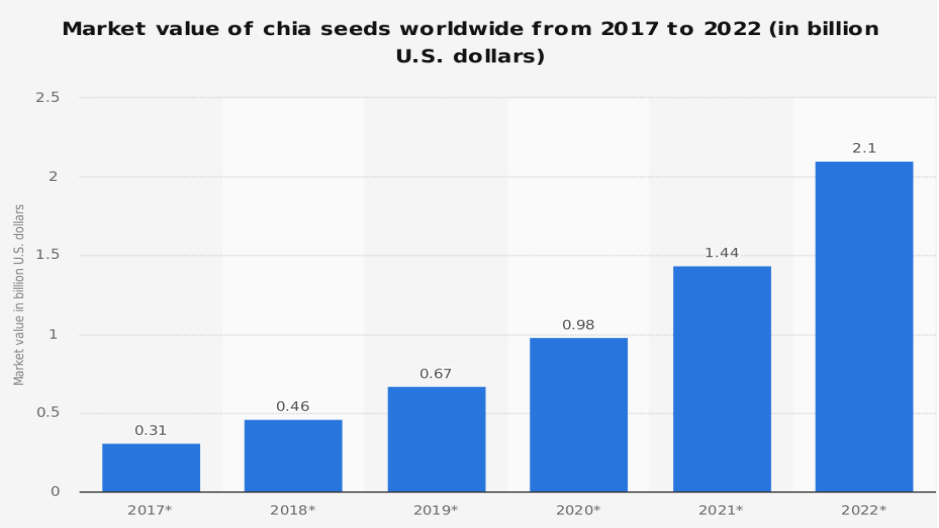

Figure 1.2. Statistical Market Value of Chia Seeds Worldwide from 2017 to 2021 Reported by Statistica Research Department on May 2018

In addition to this, nutritional quality of chia seed and oil could enhance the demand for it, all over the world. For people who suffer from coeliac disease and the ones who need protein rich diet can get advantage by integrating chia seed into their diets.

1.2. Extraction of Oil

In order to obtain valuable edible oil from seed sources, different pre-treatment processes and extraction methods are available. Prior to oil extraction, specifications of oil, ingredients, conditions in laboratories or industrial scale, equipments, further usage area of oil, chemical and physical properties of edible oil should be analyzed in detail.

There are various types of extraction approaches. Some of them could be considered as traditional which come from ancient times whereas, some of them might be named as conventional (Yusuf, 2018).

1.2.1. Traditional Extraction Methods

In old times, people tried to get beneficial oil from different plant and seed sources by primitive practices. They compressed the main product by stones or basically, performed milling. These old methods had some drawbacks in terms of time and amount of oil extracted (Yusuf, 2018).

1.2.2. Conventional Extraction Methods

Nowadays, conventional extraction methods are preferred to be used in terms of high oil yield, efficiency and broad usage perspective. By using solvent like hexane, ethanol or petroleum ether, oil inside the seed is extracted. Amount of solvent, type of seed, suitability of the solvent for seed and solvent-solute interactions, all affect the oil yield. Sometimes, for solvent extraction, rotary evaporator can be used to remove the solvent used. In addition to that, basic maceration can be applied by stirring solute and solvent, then, finally, by evaporating solvent at its boiling point with the help of incubators in laboratory condition (Yusuf, 2018).

Another solvent used specific extraction method can be named as soxhlet. Soxhlet extraction is a very useful tool for preparative purposes in which the analyte is concentrated from the solid matrix as a whole or separated from interfering substances. Conventional Soxhlet extraction remains as one of the most relevant techniques in the oil extraction field. In conventional Soxhlet method, the sample is placed in a thimble-holder and during operation is gradually filled with condensed fresh solvent from a distillation flask. When the liquid reaches an overflow level, a siphon aspirates the whole contents of the thimble-holder and unloads it back into the distillation flask,

carrying the extracted analytes in the bulk liquid (Yusuf, 2018). The whole operation is repeated until complete extraction is achieved. This performance makes Soxhlet, a hybrid continuous–discontinuous technique. Since the solvent acts stepwise, the assembly can be considered as a batch system; however, as the solvent is recirculated through the sample, the system also bears a continuous character (Sutar et al., 2010). This method is generally used to extract oil or fat portion from various food products (Nafiu and Adeyemi, 2017). On the other hand, as a drawback of this method, soxhlet apparatus may use excessive amount of solvent due to this cycling mechanism which can last for 6-8 hours. This can be reported as the main disadvantage of this extraction method (Zhang, Lin and Ye, 2018).

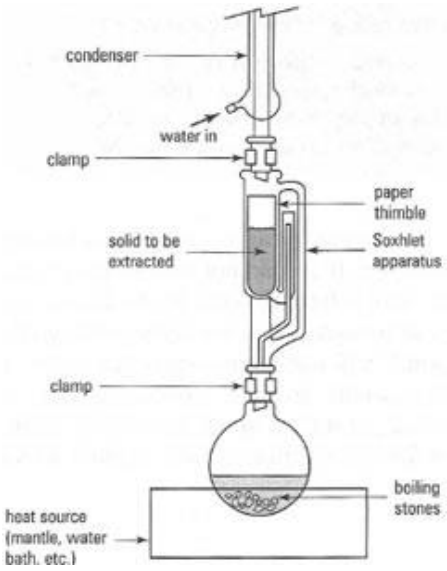

Figure 1.3. Schematic Representation of Soxhlet Apparatus

Oil can also be obtained by using mechanical aids. For instance, low oil containing seed can be exposed to high pressure or pressing. By this way, oil can be forced to release from the cell structure. Screw pressing or hydraulic can be given as examples (Arisanu, 2013). Applied external force may provide increase in oil yield. Therefore, for large scale edible oil industries, mechanical aided processes might be preferred. In

addition to this, this method requires less time, energy and amount of solvent and generate qualified oil (Carr, 1976; Kirk-othmer, 1979). Pressing method can be divided into two main groups, namely hot and cold pressing. For hot approach, as its name implies, high temperature and pressure are needed in order to remove the desired oil from the seed. For the latter approach, moderate temperatures and pressures are used to obtain enough edible oil. When two methods are compared, it is seen that cold pressing technique might have the drawback of extraction at low temperature, leading to a lower yield (Yusuf, 2018).

1.3. Antioxidants

Antioxidants are very important and beneficial weapons against free radicals in human body (Yadav, Kumari, Yadav, Mishra, Srivatva and Prabha, 2016). In other words, antioxidants are the compounds which can prevent the oxidation of substrates susceptible to oxidative reactions. They can detect the compound which they are targeted to deactivate. Even at very low concentrations within the cell, they could show effective activity against free radicals.

Besides this, people can be exposed to these compounds from their surroundings like sunlight, cigarettes, airless working rooms, and environmental pollution. (Antioxidant: In Depth, 2013). Specifically, in plant-based food sources like fruits, vegetables and spices, antioxidants are found in abundant amounts. (Yadav, Kumari, Yadav, Mishra, Srivatva and Prabha, 2016).

Furthermore, antioxidants can be explained by their relations with the oxygen. They can switch the oxygen from detrimental state to unoffending one. By this way, antioxidants could provide indirect contribution for the protection of the cells. This property refers to the principle of being an electron or a hydrogen donor (Atta, Mohamed and Abdelgawad, 2017).

In human body, antioxidants can show primary defective mechanisms and prevent undesired oxidative reactions. In this way, they might provide long term stability and intracellular calmness. (Yadav, Kumari, Mishra, Srivatva and Prabha, 2016).

Antioxidants get oxidized to block the oxidation of free radicals. Chain-breaking and preventive actions can take place for their activities (Atta, Mohamed and Abdelgawad, 2017).

For the chain-breaking mechanism, due to being free, radicals can turn to secondary phase. Changes could continue until termination phase is reached. At the end of this stage, formed radicals are stabilized by the help of antioxidant compounds like vitamin E & C or β-carotene. Since these antioxidants contribute to chain breaking and stabilizing the radicals, this mechanism is named as chain-breaking mechanism. Unlike this mechanism, in prevention mechanism, antioxidants start to affect the free radicals in the beginning, which is called as initiation in oxidation process. Furthermore, preventive antioxidants may stabilize iron or copper radicals (Atta, Mohamed and Abdelgawad, 2017).

In food industry, antioxidants are also preferred to be used in different food sources for preservative purposes. In order to retard decomposition, increase the functionality of packages and extend the shelf life of food products, natural and synthetic antioxidants may be used.

There are different types of antioxidants. Based on the name of reactions involved, existence in the nature, mechanisms providing, antioxidants are divided into groups as natural antioxidants, nutrient-derived antioxidants, antioxidants which can bind metals or enzyme origin antioxidants (Atta, Mohamed and Abdelgawad, 2017).

1.4. Emulsions

Emulsions are colloidal systems which constitute of two phases, oil and water. Generally, these colloidal structures might be in the form of oil in water (O/W) or water in oil (W/O). Based on the size of droplets, emulsions can be categorized as nano, micro and macro emulsions. Besides the difference in particle sizes, different emulsion systems may also represent different physical and chemical properties. Emulsions contain continuous and dispersed aqueous phases.

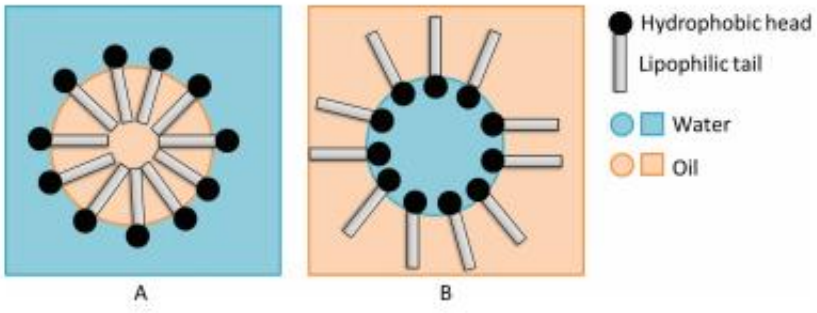

Figure 1.4. Schematic Representation of Oil in Water (O/W) and Water in Oil (W/O) Emulsions.

1.4.1. Nanoemulsions

Among the colloidal systems, nanoemulsions are the systems which have tiny particle size. Because of the small size of the droplets, functional groups, aroma components or other agents can be preserved and encapsulated within nanoemulsions effectively (Salem and Ezzat, 2018). As their names imply, particle size range of nanoemulsions can reach approximately up to a few hundred nanometers. Nanoemulsions are accepted as kinetically stable and thermodynamically unstable systems (Salem and Ezzat, 2018).

The most obvious contribution of nanoemulsions is increasing the effectiveness of lipophilic compounds like antioxidants, vitamins and phenolic compounds (Chu et al., 2007; Sagis, 2015). As in all colloidal multiple phase systems, there are continuous and dispersed phases in the nanoemulsions. Continuous phase occupies higher space by volume and create a framework for the dispersed phase so that dispersed phase can be distributed. Dispersed phase constitutes the lower percentage by volume (Çınar, 2017).

Because of the advantage of small particle size of nanoemulsions, they are preferred to be used in transparent food systems. On the other hand, depending on the type of emulsifier or surfactant used in the system, emulsion preparation method, concentration of oil and water used, size of the nanoemulsions could be changed. In addition to these, nanoemulsions have competitive edge for being resistant to flocculation, phase separation due to gravity and droplet join. For this reason, nanoemulsions are known to exhibit kinetic stability (McClements, 2010).

1.4.2. Macroemulsions

The other main emulsion group is known as macroemulsions. Like nanoemulsions, macro scaled emulsions are known to be thermodynamically unstable systems in food science. However, in addition to thermodynamic unstability, macroemulsions are also kinetically unstable due to rapid phase separation. This means that, when oil and water phases with the addition of surfactant are prepared, a milky and turbid appearance is obtained. Furthermore, it is inevitable that phase separation due to different mechanisms is observed. When macroemulsions are taken into consideration from a theoretical point of view, they may be described as distribution of dispersed droplets which have diameter larger than 0.1μm within continuous phase. Generally, they have a droplet size between 0.1 to 100μm (Saifullah, Ahsan & Shishir, 2016). However,

they might not be preferred in transparent liquid food systems (Sharma and Shah, 1985).

1.4.3. Microemulsions

The last major classification of emulsions is called microemulsions. Unlike nano and macroemulsions, microemulsions are thermodynamically stable colloidal systems. Mean particle size of these emulsion systems is within the range of 10-100 nm (Gupta, Eral, Hatton and Doyle, 2016). Since microemulsion systems are stable colloidal systems, phase separation, coalescence or Oswald ripening may be observed rarely. Under favor of advantages in stability and particle size, microemulsions might be preferred in food, pharmaceutical and chemistry areas. Specifically, oil dominant food products may require microemulsion systems in order to keep their homogeneity (Paul and Moulik, 2001).

1.5. Nanoemulsion Formation

To create nanoemulsions, two different techniques are used. These include low and high energy treatments. (Tadros et al., 2004; Acosta, 2009; Leong et al., 2009; McClements, 2010; Qian and McClements, 2011; Adheeb Usaid et al., 2014). For low energy methods, internal chemical energy of the system is used in order to obtain emulsion systems. In high energy methods, external mechanical aid is required to produce extra energy for emulsification. Disruptive forces are produced so that bigger droplets can be divided into smaller and stable ones. In order to successfully create them, exterior forces should be more powerful than the holding forces between droplets which are already created in the emulsion (McClements, 2010).

1.6. Nanoemulsion Formation Methods

1.6.1. Low Energy Treatments

Emulsion inversion point (EIP), phase inversion temperature (PIT), phase inversion composition (PIC) and spontaneous emulsification (SE) approaches are the known low energy methods (McClements, 2010). Low-energy processes occur under laminar and low-energy conditions, which is of interest from an economical perspective (Santana, Perrechil and Cunha, 2013). In addition to these, low emulsion emulsification methods are attractive nowadays due to their wide application and mechanical easiness in terms of formulation and stability aspects. This method is primarily controlled by the physicochemical behaviour of the surfactants and requires careful selection of surfactant and co-surfactant (Sharma, Bansal, Visht, Sharma and Kulkarni, 2010).

1.6.1.1. Emulsion Inversion Point (EIP)

Alteration of oil in water (O/W) to water in oil (W/O) emulsion system or vice versa is known as EIP treatment. As its name implies, by addition of water or oil into a system where oil or water is the continuous phase respectively, transition between continuous and dispersed phases could be achieved. When added dispersed liquid phase is reaches a critical amount, inversion mechanism occurs automatically. On the other hand, type of surfactant used or surfactant to phase ratio (SPR) or rate of the dispersed phase addition to continuous phase may affect EIP catastrophically (McClements, 2010).

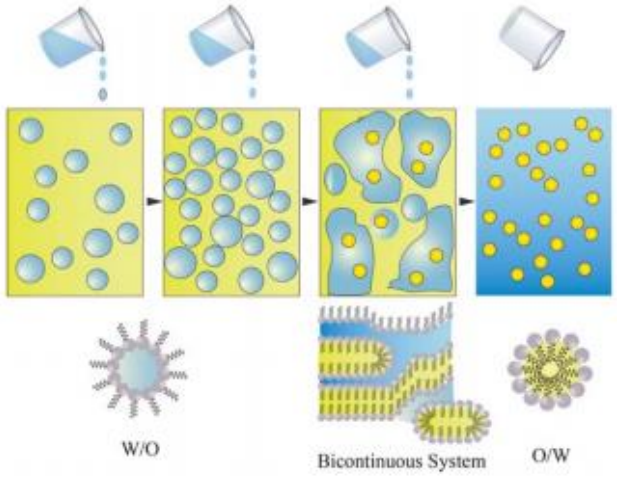

Figure 1.5. Illustration of Emulsion Inversion Point (EIP) and Phase Changes.

1.6.1.2. Phase Inversion Temperature (PIT)

By changing the temperature, structural conformation of molecules or emulsifier chemistry might show variation. For a specific temperature, continuous and dispersed phases could alter their places like the emulsion inversion point approach. However, PIT might have some disadvantages like formation of coalescence due to temperature increase. If nanoemulsions are prepared at lower PIT, smoothness can be obtained in the system. Nevertheless, PIT method may not be the correct approach; especially for processes where it is necessary to use high temperatures like pasteurization or sterilization (McClements, 2010).

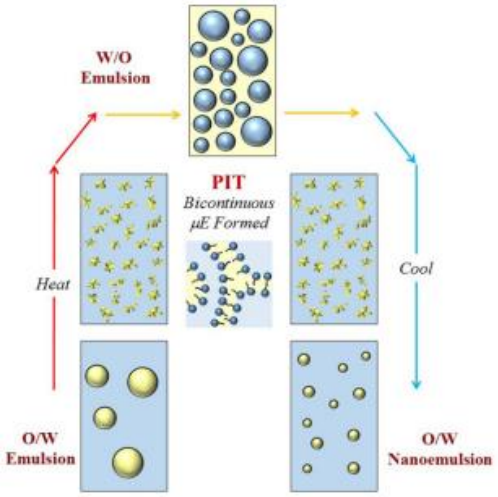

Figure 1.6. Representation of PIT and Phase Inversion Due To Temperature Changes.

1.6.1.3. Phase Inversion Composition (PIC)

Mechanism related to PIC resembles the PIT with one difference. Instead of temperature change, molecular sequence within the emulsion system may change by interior or exterior parameters. For instance, integration of ionic molecule to the emulsion may lead to change in the charge balance of the surfactant or emulsifier. In addition to that, external salt establishment might result in phase inversion from O/W to W/O (McClements, 2010).

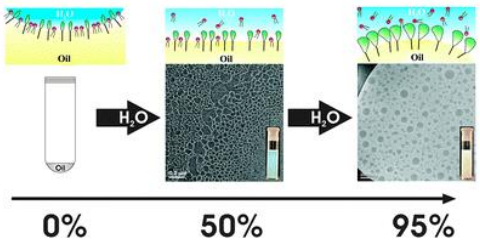

Figure 1.7. Visual Representation of Phase Inversion Composition (PIC) and Possible Composition Change.

1.6.1.4. Spontaneous Emulsification

For spontaneous emulsification, oil and surfactant phases are mixed together mostly with the help of a stirrer and then this dispersed phase is dropped into the continuous water phase. Therefore, it can be implied that for O/W emulsion systems, this method is more suitable. For spontaneous emulsification, type of fluids used, their solubility with each other, rate of stirring, chemical and physical properties of surfactant, surfactant to oil ratio (SOR) are all factors affecting droplet size and stability of emulsions. When mechanism that underlies the system is investigated, it can be said that for SE method, firstly dispersed and continuous phases are found together as a unique phase. While mixing is proceeded, surfactant used could pass through continuous water phase so that it can act as a bridge between water and oil with the help of turbulent strength. Moreover, water soluble molecules represented in dispersed phase start to deport themselves from whereabout into water phase. This motion can create a huge interfacial area between oil and water. Consequently, proper oil droplets are structured within continuous water phase spontaneously (McClements, 2010).

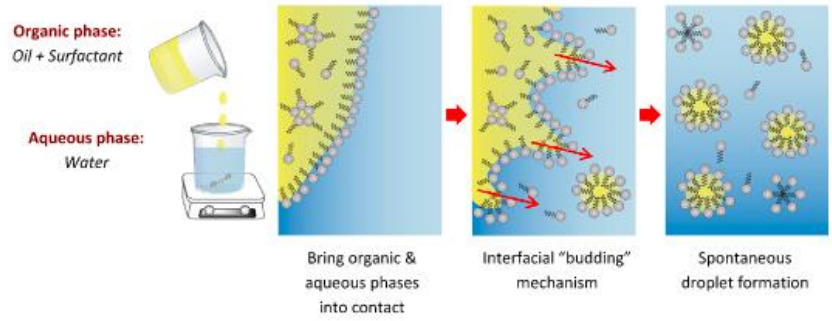

Figure 1.8. Illustration of Mechanism Which Underlies Spontaneous Emulsification.

1.6.2. High Energy Treatments

For high energy methods, an exterior mechanical device is needed to give extra energy for system to give rise to small and stable droplets. Microfluidizers, homogenizers

worked with high pressure and ultrasound-based devices use high energy for emulsification. They are used in order to obtain small droplets containing emulsion systems in the food industry (McClements, 2010). Although high-energy emulsification is traditionally used in industrial operations because of the flexible control of emulsion droplet size distribution and the ability to produce fine emulsions from a wide variety of materials, they have undeniable disadvantages. They have higher costs due to additional external mechanism. Furthermore, increase in device-related local temperature might decrease the activity of the emulsifier. In addition to this, application of very high pressures can result in droplet re-coalescence (over-processing) and a consequent destabilization of the emulsions (Santana, Perrechil and Cunha, 2013).

1.6.2.1. Microfluidizers

One of the most commonly used nanoemulsion formation devices using high energy approach are microfluidizers. Microfluidizers have some compartments to mix oil and water phase, and to bring them into a unique phase on which oil droplets are distributed by nano scale. Firstly, oil and water phases come up, then they start to interact with each other. This takes place in an interaction chamber. The crucial point of an microfluidizer can be the iterative passage of oil and water mixture from there. By this way, nano-scaled tiny droplets could be obtained (McClements, 2010). In addition to that, oil-water mixture is sent to the interaction chamber where very high pressure of 500-20,000 psi is applied to gain stable nanoemulsions (Borthakur, Boruah, Sharma and Das, 2016). Furthermore, by increasing the pass times and surfactant amount or applying a higher pressure, droplet size of oil phase within the continuous phase may be minimized.

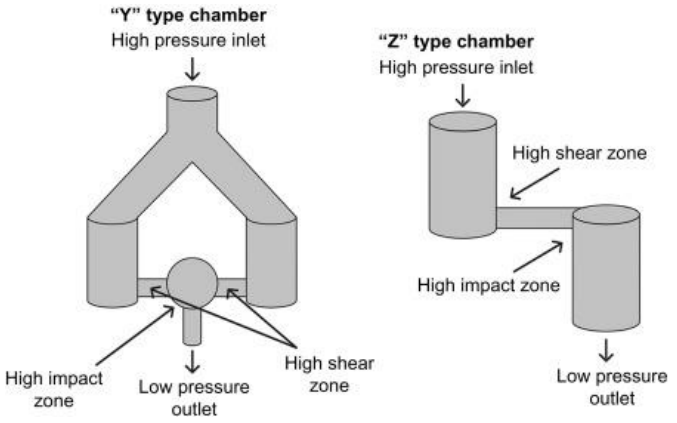

Figure 1.9. High Energy Microfluidizer Types in Food Industry.

1.6.2.2. High Pressure Homogenizers

Among the high energy based nanoemulsion preparation methods, high pressure homogenizer is the most popular and efficient method to generate tiny droplet containing nanoemulsions. For this reason, it is the most convenient method in different food processes, specifically for oil-based beverage industry. Being different from other methods, homogenizers can have the mechanical ability to break bigger droplets into smaller ones by enhancing shear and turbulent forces and creating cavitation through pump containing valve (McClements, 2010). This valve contains broad and narrower orifice. When the diameter of orifice decreases, pressure on oil and water mixture increases up to 500-5000 psi. By decreasing the diameter of orifice, increasing applied pressure or time and using various homogenizers, droplet sizes of final emulsions can be decreased (Shakeell etal.,2008).

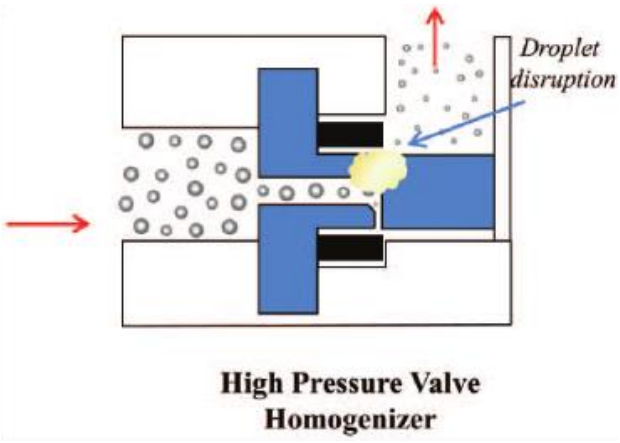

Figure 1.10. Schematic Representation of HPVH.

1.6.2.3. Ultrasonic Homogenizers

As its name implies, ultrasonic homogenizers are based on the usage of ultrasonic waves to create stable and tiny droplets by using frequencies above 20-25 kHz. Although high pressure homogenizers are used abundantly in food industry, for small-scale food researches, sonicative methods are widely used. In terms of advantages related to using, arranging experiment parameters and manipulating the device, these kinds of homogenizers are given place in food science. Since ultrasonic devices are manual and easy to control, by changing power and time used, adding more surfactant or arranging surfactant to oil ratio (SOR), smaller droplets can be obtained (McClements, 2010). In spite of the abundance of other high energy-based devices, sonication has winning edges in terms of equipment cost and ease of handling (Kentish, Wooster, Ashokkumar, Balachandran, Mawson and Simons, 2008).

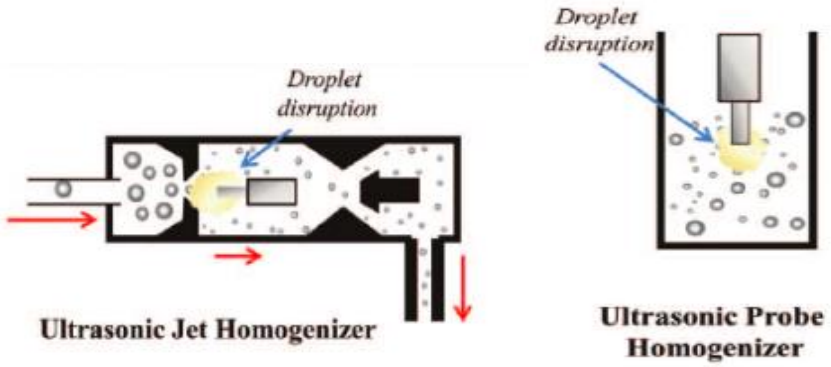

Figure 1.11. Schematic Representation Different Ultrasonic Homogenizer in Food Science.

1.7. Role of Surfactant in Emulsification

In high and low energy emulsification methods, role of surfactant selected is very important. Generally, type of surfactant, its compatibility for the oil and emulsion system and acceptability for the emulsification method are essential points.

Surfactants are amphiphilic compounds that reduce the interfacial tension between the oil and water interface, favoring droplet disruption during emulsion formation and preventing the droplets from aggregation and re-coalescence (Jafari and Bhandari, 2007). The type and concentration of surfactants used are crucial factors in determining the droplet size distribution and stability of the emulsion. A wide range of surfactants is considered as food grade, including small molecular weight surfactants, phospholipids and biopolymers (Krog and Sparso, 2004). Small molecular weight surfactants are generally more efficient in emulsion stabilization than biopolymers. They are rapidly adsorbed on to the freshly formed surface of the droplet and stabilize the new interface in milliseconds, preventing re-coalescence of the droplets. Small molecular weight surfactants are successfully used in both low- and high-energy processes. Biopolymers are slower emulsifiers that can only be effectively used in high-energy emulsification, specifically in systems with a longer

residence time, such as colloid-mills or multistage high-pressure systems (Jafari and Bhandari, 2007). Surfactants are classified according to the hydrophilic– lipophilic balance (HLB). Generally, W/O emulsions are produced using surfactants with a low HLB (3–8), while O/W emulsions are created with surfactants with a high HLB (8–18). Small molecular weight surfactants may be classified according to charge, including cationic, anionic, non-ionic or zwitterionic. Examples of cationic and anionic surfactants include lauric arginate and biosurfactants (rhamnolipid), respectively (Kralova and Sjöblom, 2009). Non-ionic surfactants, such as sorbitan fatty acid esters (spans), polyoxyethylene sorbitan fatty acid esters (tweens), sugar esters and monoglycerides, are extensively used in the food and pharmaceutical industries (Leser, Sagalowicz and Watzke, 2006). Mono- and diglycerides are approximately 70% of the total emulsifiers used in the food industry (Garti, 2001). They can be used to cover the increased surface area of the fat globule derived during milk homogenization and induce emulsion destabilization during freezing/aeration of ice cream process due to protein and surfactant competition adsorption (Lal, O'Connor and Eyres, 2006). Zwitterionic surfactants, such as phospholipids, are of interest in food applications due to their ‘‘Generally Recognized as Safe’’ (GRAS) designation. However, their use in low-energy emulsifications is limited, and the addition of other surfactants and co-surfactants is necessary to produce microemulsions (Flanagan and Singh, 2006).

Unlike mentioned ionic surfactants, Tween and Span groups show nonionic chemical structure and have a wide range of application in emulsion-based food industry. Therefore, they are more compatible for emulsion systems, specifically for the ones prepared with low energy methods. Chemically, sorbitan esters are known as Span groups. They are marketed under Span product name and produced by the dehydration of sorbitol. In addition to this, in simple terms, Tweens are ethoxylated Spans. Tween groups are quite hydrophilic in nature and soluble or dispersible in water or dilute solutions of electrolytes. Therefore, Tweens based on unsaturated and branched chain fatty acids act as effective oil in water emulsifiers. Moreover, hydrophilic lipophilic

balance (HLB) value of surfactant and its appropriate chemical structure are vital for emulsion systems. For instance, surfactants with an unsaturated alkyl chain have an increased affinity for oils containing unsaturated bonds. In this case, usage of Tween 80 can be ideal to emulsify most of the seed-based oil.

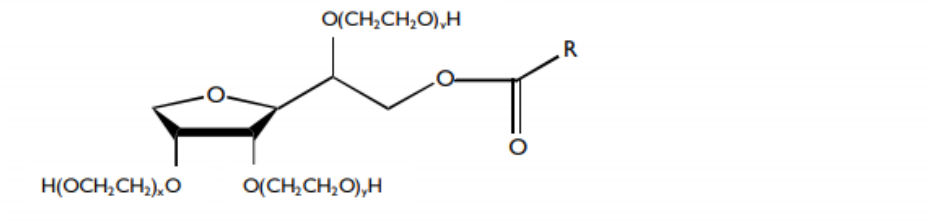

Figure 1.12. Chemical Structure of Polyethoxylated Monoester (Tween) Surfactants.

1.8. Bioavailability and Bioactivity of Emulsions

As it has been mentioned in previous parts, antioxidants have undeniable strong effects on protection from free radical reactions and occurrence of undesired oxidation. By the help of diverse mechanisms, they have the ability to act as guards against free radicals which are mainly responsible for chronic diseases, cancer and metabolism disorders (Zorzi, Caregnato, Moreira, Teixeira and Carvalho, 2016).

Significant amount of plant and animal-based food sources have antioxidative compounds. However, when storage and consumption conditions or other environmental effects are taken into consideration, it is ineluctable to be faced with oxidation related problems by indicators of rancidity, color and texture alterations. Thus, it is preferred to create emulsion or encapsulation systems to store these compounds. In comparison with encapsulation, emulsion systems can be obtained in

larger range of particle size with various high and low energy approaches. On that account, emulsion systems may be ideal supporters for both lipophilic and hydrophilic food products. Furthermore, emulsions, most particularly, nano or micro-colloids might be assigned as effective carriers of antioxidants, nutritive additives, flavors or essential components (Friberg et al, 2004; McClements, 2005; Leal-Calderon et al, 2007).

Bioavailability of sensitive lipophilic components like antioxidants or unstable essential oil or flavor systems are enhanced significantly in nanoemulsions. They can be trapped within the colloidal system by inhibiting undesired interactions with other ingredients (Khaled, Ramadan and Ashoush, 2014). By this way, presence of active compounds as an emulsion system could be easier, and controlled oscillation from the colloidal system might be achieved. In addition to these, due to the small droplet sizes of emulsion systems, more homogeneous and transparent food systems as well as advanced nutritional features can be obtained. Nominately, phase separation like Ostwald ripening, gravitational separation, micelle formation, which are the main problems in both oil and water containing products, can be minimized (Khaled, Ramadan and Ashoush, 2014). Consequently, bioactivity and availability of active compounds, antioxidants and flavors may be improved by constitution of emulsion systems (Jochen et al, 2009).

1.9. High Hydrostatic Pressure

When the historical development of food processing techniques is investigated, it is found that, the idea of using pressure as a food processing method dates back to ancient times. Primitive researches into the application of HHP for milk preservation began when Hite demonstrated that the shelf life of milk and other food products could be extended by pressure treatment (Balasubramaniam et.al., 2015) in 1899. However, unavailability of suitable equipment hampered early applications of HHP. The advances achieved in ceramics and metallurgical industries in the use of high-pressure techniques during the 1970s and 1980s has led to the possibility of treating food by

this method at industrial level. The first commercial HHP-treated products (high acid jam) appeared in the market in 1991 in Japan, where HHP is now being used for products such as dairy foods, fruit juices, jams, sauces, rice, cakes and desserts etc. (Tao et al., 2014; Muntean et al., 2016).

Before the invention of this novel technology, conventional food-processing strategies had relied on temperature dependence as a way to ensure prolonged shelf-life and nourishment security. However, the utilization of specifically high temperatures is commonly known to cause undesirable changes in the prepared food systems. These changes may affect both sensory properties of food products and nutritional features of them. Furthermore, important ingredients of food products like vitamins, minerals, exclusive essential oils or aromas might be damaged. Moreover, texture which is one of the most appealing characteristics of any specific product could also be affected in out of favor manner. By considering all of these possible consequences, it is ineluctable that scientists and consumers have started to look at alternative, novel and better preservation techniques. Change in health concerns and requirement of meeting the nutrition-based needs of today's human being, have enhanced the application area of HHP (Parekh, Aparnathi and Sreeja, 2017). Especially after 1994, freshness, nutritive aspects and health issues have become the focus in food processing. Due to this, non-thermal advanced technologies have gained more attention. Profitably, HHP application have found wider area of utilization (San Martín, Barbosa-Cánovas and Swanson, 2002).

According to Yordanov and Angellova (2010), several physical and chemical changes result from the use of pressure. Physical pressure throughout pressure processing brings about a volume decrease and an increment in temperature and energy. The rationale for the use of HHP is in conformity with the three elements of physical and chemical principles (Yordanov and Angelova, 2010). Therefore, during HHP application, LeChatelier’s and isostatic principles are valid. According to

LeChatelier's principle, any phenomenon such as chemical reaction, conformational change, stage transition, that is conducted by a decline in volume is improved by pressure. At consistent temperature, an expansion in pressure expands the degrees of ordering of molecules of a substance. In this manner, pressure and temperature apply opposed forces on molecular structure and chemical reactions. In addition to this, according to isostatic principle, food items are condensed by even pressure from each angle and after that, they came back to their unique shape when the pressure is discharged. The items are condensed freely of the item size and geometry because transmission of pressure to the center is most certainly not mass and time dependent. Therefore, the procedure is minimized (Ginsau, 2015; Martínez-Monteagudo and Balasubramaniam, 2016).

In high pressure processing, the pressure vessel is filled with a food product and pressure is applied for a desired time following which it is depressurized. The time required to develop pressure in the vessel is influenced by the compressibility of the pressure medium and the nature of the food material. In most cases, water is used as the pressure transmitting medium. Presence of air in the food increases the pressurization time, since air is considerably more compressible than water. The pressure is applied isostatically. Therefore, pressure remains uniform in the product and the entire product undergoes the same treatment (Chawla, Patil and Singh, 2010). High pressure process is characterized by three parameters: pressure (p), temperature (T) and exposure time (t). Before application of HHP treatment to any food source, these three parameters are decided according to chemical and physical features of product (Naik, 2016). In a regular HHP system, temperature indicator, pressure vessels, pressing apparatus, pressure and temperature reader legend are found. Inside the device, 100 MPa to 1000 MPa pressure is applied to the food which is found inside the aqueous system for laboratory conditions (San Martín, Barbosa-Cánovas, and Swanson, 2002). In order to get clear results, pressures more than 1200 MPa could be chosen. In addition, HHP when applied at high degrees like 600-800 MPa is successful

for inactivation of enzymes in beverage and juice industry (Yamamoto, 2017). Commercial high hydrostatic pressure processing device is given in **Figure 1.13.**

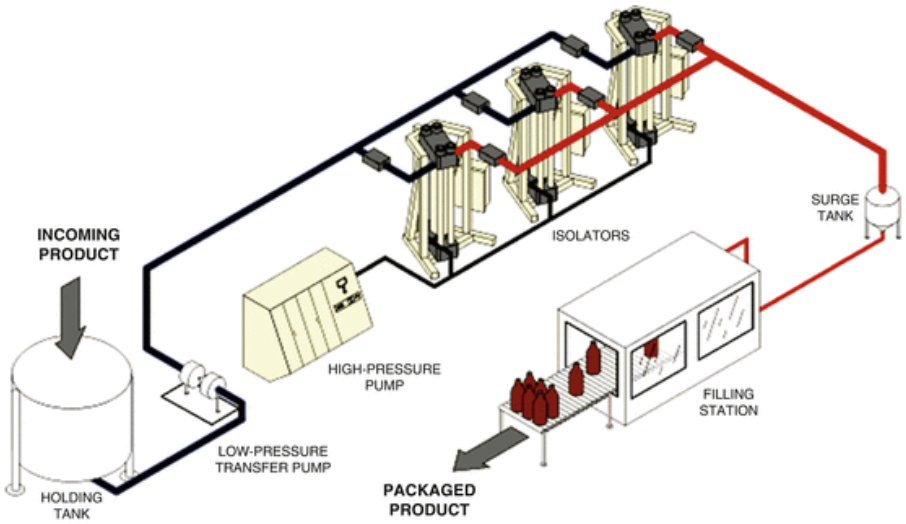

Figure 1.13. Commercial High Hydrostatic Pressure Device in Food Industry.

There are other important commercial applications of HHP for food products. They can be summarized as pasteurization and sterilization of milk, meat, vegetable, fruit, salad dressing and yoghurt. It is also revealed that HHP treatment has effective results on various food products (Yamamoto, 2017). These effects are evaluated in terms of microbial safety, physical changes in food sources, functionality and nutritional characteristics. For instance, it is proven that HHP treatment inactivates microbes lethally or sub-lethally while minimizing the degradation of color, texture, appearance and flavor. As a result, it can be deduced that HHP application helps in obtaining

minimally processed high quality safe food products. Due to these reasons, HHP is preferred in meat, dairy and fishery industry as well as fruit and vegetable sources (Muntean, Marian, Barbieru, Catunescu, Ranta, Drocas and Terhes, 2016).

Furthermore, HHP treatment contributes to functionality and nutritional characteristics of food products. In order to pasteurize meat, HHP is a better option than food additives or lactic acid bacteria which leads to unfavorable acid or polysaccharide forming undesired pH. Besides all these application areas, demands for HHP in different food sources are increasing rapidly (Yamamoto, 2017).

HHP treatment may induce some microscopic changes in food biopolymers. By HHP process, non-covalent bonds like starch, protein or nucleic acids may face with some structural changes like denaturation, coagulation or gelatinization. Most of the time, these changes are desired ones and they might contribute to shelf life and in preservation of food products. For example, starch is one of the major components and acts as a gel forming texture modifier. Within the water containing environment, starch gelatinized in the presence of heat treatment. Similarly, HHP treatment can contribute to starch gelatinization. When the effect of pressure and heat are investigated, it can be concluded that enzymatic degradation and visco-elastic behavior of HHP treated starch is upgraded. Moreover, retrogradation which is one of the important events for starch products and linked to recrystallization and stiffness, can become more rapid when HHP treatment is applied (Ginsau, 2015). One another important usage area of HHP treatment is in lipids. Specifically, for chocolate-based food products, lipids and morphological changes in lipids are essential. HHP treatment can induce phase transition of lipids. Also, high pressure can control the thawing and freezing mechanisms (Yamamoto, 2017). Another application of HHP is in grain industry. HHP treatment may reduce allergens via extraction. It is known that,

hypoallergenic cooked rice and wheat bread were developed by HHP based extraction of rice and wheat allergens (Yamazaki and Sasagawa, 2017).

Furthermore, HHP treatment may also be used in fruit and vegetable industry. HHP processed fruits and vegetables have longer shelf life and more fresh attributes than conventional treated ones. For example, HHP- processed jam contains significant amount of flavor components like linalool and 2- methyl butyric acid. Besides, HHP application might enhance the amount of food components such as vitamin C or butyric acid (Yamamoto, 2017). Additionally, HHP treatment plays an important role in enzyme inactivation. Combination of high pressure and temperature can increase the effect of inactivation of enzymes like lipoxygenase, alkaline phosphatase, pectin-methylesterase, polyphenoloxidase. Due to inactivation of enzymes, sensory properties and functionality of food products might be enhanced (Yamamoto, 2017).

The effect of HHP on oil yield before extraction and particle size of emulsions are not investigated so far. Therefore, it is not possible to know the mechanism and possible effects of high pressure in advance. However, similar outcomes for chia oil yield may be obtained from the studies related to extraction of active compounds from different sources. For instance, according to the study related to extraction of flavonoids from propolis by using HHP, there is a significant effect of high pressure on extraction yields of flavonoids when compared to classical extraction at room temperature. Due to disruption of cell structure and an increase in more active compounds, extraction yield increases (Shouqin and Changzheng, 2005). On the other hand, there is no scientific study to foresee the effect of possible HHP on particle size of emulsions.

High hydrostatic pressure application has other significant advantages compared to conventional processing techniques. As the most valid one, HHP does not require specific product shape and geometry for usage. For this reason, it can be available for all types of food systems (Parekh, et al., 2017). Moreover, HHP device does not need

strict and continuous temperature control system. Furthermore, since pressurization application is performed at room temperature, consumed energy for the process can become minimum even high pressures are reached. Furthermore, flavor, nutritional value, color, aroma and texture of food products are preserved. By this way, consumer acceptance is also increased. Unlike conventional treatment methods, processing time is reduced by using high hydrostatic pressure application since pre-treatment for food or external temperature control are not required. Moreover, HHP treatment does not lead to formation of toxicity since only water is available within the pressure application region. For this reason, this treatment can be considered as ecologically friendly.

The Food and Drug Administration (US FDA) has approved HHP as a non-thermal pasteurization technology that can be used to replace traditional pasteurization in the food industry. United State Drug Administration (2012) has requirements E. coli O157:H7 as the indicator strain for reprocessing, an HHP process that achieves a 5-log E. coli O157:H7 reduction should be enough for product produced to ensure microbial safety. The Food and Drug Administration (FDA) has approved HHP for production of low acid foods and many food processing industries in 2009. Japan and European continent have already started using this technique for preparation of various food items (Houska et al., 2006). In Europe, HHP is regarded as a novel technology subject to the Novel Food Regulation. The European Community (EC) recently founded a major multinational research project on HHP to make a real assessment of the potential of high-pressure technology for commercialization (Huang at al., 2017).

Recently, 31% of industrial meat products, 35% of vegetable products, 12 % of juice and beverages, 14% of sea food and fish products are being produced by HHP treatment commercially. In addition, as a notable example for this study, HHP has been applied to salad dressing products which contain emulsion systems (San Martin et. al., n.d.).

1.10. Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is one of the most powerful and versatile analytical techniques that can be applied to liquid and/or solid materials and has become increasingly popular in the field of food science for the evaluation and the analysis of several foods, such as beverages, oils and lipids, vegetables, meat, and dairy products. Although initial NMR applications in foods were limited to low resolution NMR analysis of moisture, at the end of the 20th century, high-resolution studies of liquid and solid-state matrices also gained importance for several purposes. These included classification (Dais, Hatzakis, 2013; Marcone et al., 2013; Spyros & Dais, 2013), quality control (Marcone et al., 2013), sensory evaluation (Malmendal et al., 2011), structural characterization and compositional analysis (Bertocchi & Paci, 2008; Cheng & Neiss, 2012; Spyros & Dais, 2009, 2013; Vlahov, 1999), understanding molecular mechanisms and interactions of food components (Fernandes, Bras, Mateus, & ´ Freitas, 2015; Leydet et al., 2012; Tiziani, Schwartz, & Vodovotz, 2008), as well as the investigation of nutritional approaches to health (Ramakrishnan & Luthria, 2017).

For food application mostly low-resolution systems are used and Time Domain NMR Relaxometry analysis are performed. Molecular interactions, moisture/oil distribution, hydration, crystallization, adulteration can be investigated with this technique. For high moisture food products in order to foreseen the shelf life, observe the possible ongoing reactions and mobility of water molecules and interpret the effects of various heat treatments, nuclear magnetic resonance can be the alternative way to find important outputs (Fernandes, Bras, Mateus, & ´ Freitas, 2015; Leydet et al., 2012; Tiziani, Schwartz, & Vodovotz, 2008). In addition, in dairy industry, denaturation, water and fat related properties like water and fat holding capacities, NMR technique may be a good choice. Furthermore, solid fat content which is one of the primary characteristics in fat based food products or crystal structures can be examined by nuclear magnetic resonance practices (Ersus et al., 2010; Oztop et al., 2012). As

emulsion being a 2-phase system interaction between oil and water could be examined using these techniques (Duynhoven, Voda, Witek and Van As, 2010).

In NMR, electromagnetic waves which are between the range of radio-frequency (RF) are used (frequency range of 4-600 MHz). Basically, transmission and uptake of energy mechanisms take place. When NMR tool is run, a magnetic field is generated and all the protons are alligned in the direction of this field. When an RF pulse is transmitted onto this magnetic field, protons are flipped into the XY plane leading to an increase in transverse magnetization. The angle of the flip can be less than 90^o, 90^o or 180^o. The RF pulse is then removed, and protons are allowed to relax back to their original position which is in the direction of external magnetic field (B_o) (Han, Zhang, Fei and Xu, 2009). Simultaneously, a signal is generated and this signal provides information about not only the relaxation of the protons but also the decay of transverse magnetization (Todoruk, Litvina, Kantzas and Langford, 2003).

In TD-NMR relaxometry, two time constant are important. Chemical and physical reactions, possible mechanisms or structural interpretations are examined by using T_1 and T_2 time constants. These time constants are unique for each sample. Fundamentally, T1 time constant is named as spin-lattice relaxation time (Hashemi, Bradley, & Lisanti, 2010). It shows the degree of energy send by nuclei to the surrounding. T1 relaxation time depends on magnetic field strength which means that as this strength increases, T1 increases. The latter time constant T2 is denoted as spin-spin relaxation time. It is used to explain the mechanism of energy release by nucleus to the other surrounding nuclei. T2 relaxation time is generated by the exchange of energy between nuclear spins (Ersus et al., 2010). The decay of the magnetization on the XY plane back to an initial value of zero, is known as transverse relaxation time (Kırtıl and Oztop, 2016). T_2 relaxation time is determined by using the Carr-Purcell-Meiboom-Gill (CPMG) sequence. This sequence involves the application of 90^o RF pulse initially and then followed by a series of 180^o RF pulses. The purpose of using such a sequence is to improve the signal and diffusion constant efficiency by

enhancing the signal to noise ratio (SNR) (Preto, Tavares, Sebastiao and Azeredo, 2013). In addition to this, the effect of external inhomogeneities can be reduced (Argin et al., 2014).

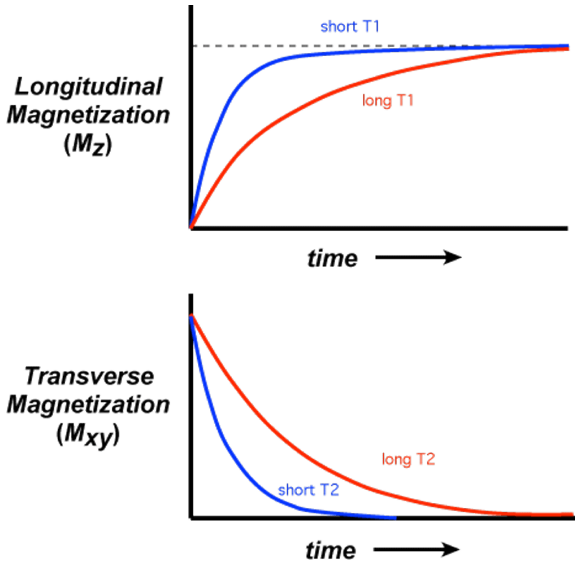

Figure 1.14. Graphical Representation of Different Longitudinal (T1) and Transverse (T2) Magnetization Concepts.

1.11. Objectives of the Study

Majority of the people hope to find more healthy, nutritious and functional food sources, and food scientists explore efficient processes for them. Novel technologies and products have caught people's attention. Nano and micro emulsion systems are used as fully operational delivery aids which meet the needs of both consumers and scientists in terms of acting as functional delivery and encapsulation devices. They can embed the nutritional and essential components and contribute to more homogeneous, transparent and valuable final food products. In this study, the objective is to design chia seed oil nanoemulsions using the low energy method of spontaneous emulsification. In addition, to obtain stable emulsions, physical characterization experiments were also conducted for different formulations. Effect of high hydrostatic pressure, surfactant to oil ratio were examined on the stability of the emulsions. Mechanism underlying the emulsions formation was also aimed to be explored using microscopy experiment.

Hypothesis of this study is that chia seed oil, in the form of a nanoemulsion, would be protected more and would have better stability.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Chia seeds of South America origin were obtained from "Nev Gıda Industry and Trade Inc. Ostim/ANKARA. They were grinded to powder form by using a coffee grinder (Bosch MKM 6000 UC). Hexane, acetic acid, methanol, DPPH reagent, Folin Ciocalteu's phenol reagent, chloroform, potassium iodide was supplied by Sigma Aldrich Laboratory Equipment Trade Corporation (St. Louis, USA).

2.2. Methods

2.2.1. Chia Oil Extraction from Chia Seed

Chia seed has significant amount of carbohydrates in its crust part and it leaches out as a mucilage when soaked in water. Firstly, chia seeds were grinded. Then, ground chia seeds were used for extraction. For extraction, two different methods, namely, *Soxhlet* and classical extraction methods were used.

2.2.1.1. Soxhlet Extraction

Soxhlet extraction was performed by using the soxhlet apparatus (EFLAB). Hexane was used as the solvent and 5 g of grinded chia seed samples were placed to the each column of the soxhlet apparatus. Oil extraction continued for 5-6 hours.

2.2.1.2. Classical Extraction (Maceration)

For this extraction process, grinded chia seed and hexane as a solvent were mixed at a ratio of 1:10 (w/v). Then, the mixture was stirred by magnetic stirrer for 1 hour at 50^{o}C. After cooling the mixture to the room temperature, it was centrifuged at 4,000 rpm for 15 minutes. As the final stage, in order to obtain pure oil, mixture was put to the incubator at 60^{o}C for 36 hours to remove the hexane.

2.2.1.3. Effect of High Hydrostatic Pressure on Oil Extraction

To test the effect of High Hydrostatic Pressure on oil yield, seeds were also exposed to HHP at different conditions.

HHP was conducted by using 760.0118 based equipment (SITEC-Sieber Engineering AG, Zurich, Switzerland). Vessel volume of the equipment was 100 mL. Internal diameter and length of pressure equipment were 24 mm and 153 mm respectively. Distilled water was the aqueous medium inside the equipment. A built in heating and cooling system was used to keep the inside temperature of the system constant (Huber Circulation Thermostat, Germany). High hydrostatic pressure was applied at 300 and 500 MPa at 30^{o}C for 5 minutes. Before applying pressure, grinded chia seed and hexane mixtures were put to the pressure tubes with a volume of 25 mL. Then it was fixed to the pressure vessel. Finally, HHP was applied to the seeds to extract the oil.

In addition to these, HHP was also applied after emulsion formation. In order to see the effect of HHP on particle size of the emulsions, 300 and 500 MPa pressure were applied at 30^{o}C for 5 minutes. HHP was applied with the same equipment. After application of high hydrostatic pressure, mean particle sizes of emulsions were analyzed. By this way, the effect of HHP on emulsion droplets were determined.

2.2.1.4. Identification of Fatty Acid Composition of Chia Seed Oil by Gas Chromatography

Gas Chromatography (TRACE 1300) was used to determine the fatty acid composition. Gas chromatography (GC) can analyze the fatty acids in their methyl ester forms (Fatty Acid/FAME Application Guide, n.d.). The followed method was the modified form of the method described by Jeon et.al. (2016). 0.25 g oil and 6 mL of 0.5 N of methanol and sodium hydroxide were mixed to produce methyl esters. Then it was heated in water bath at 80^{o}C for 10 minutes. After this step, heated oil was cooled by the help of ice for 2 minutes. At the same time, 7 mL of 14% boron trifluoride methanol was added to the solution and the mixture was heated again to 80^{o}C for 3 minutes. Finally, the solution was cooled for 3 minutes in an ice bath before the solvent n-hexane was added. After all heating and cooling, oil was heated for a couple of seconds and the top layer was separated and transferred to a vial. GC included a capillary column and ionization detector (260 flames) (Jeon, et al., 2016). As the carrier gas, Helium was used at a rate of 1.3 mL/ min. 1µl of solution was added at a split ratio of 50:1.

2.2.2. Emulsion Preparation from Chia Seed Oil by Spontaneous Emulsification (SE)

Following the extraction of chia oil, nanoemulsions were obtained by using spontaneous emulsification method. Oil samples obtained from HHP treatment and conventional extraction were used for spontaneous emulsification. First, concentration of the oil and the surfactant were determined. When the nanoemulsion studies in the literature were examined, it was observed that a carrier oil was also used in addition to the main oil to stabilize the emulsion (Bouchemal, Briancon, Perrier and Fessi, 2004). In this study coconut oil was first tested as the carrier oil. After different surfactants (lecithin and Tween 80) and carrier oil (coconut oil) trials, it was decided that chia oil was enough to obtain stable emulsions. Effect of surfactant to oil (SOR) ratios were examined to find the best options in terms of physical stabilities. SOR of

1,2,3 and 4 (w/w) were tested. Lecithin was also found to be inappropriate for the emulsions since it led to aggregation problem after combining with chia oil within the water phase.

As a result of preliminary studies, SOR was decided as 4 since it gave the best stability. Tween 80 was used as the sole surfactant. Stability was checked through storage at room temperature and 50% RH for 4 weeks. Oil concentrations were used at 0.5, 1, 1.25, 1.5, 2.5, 3 % (w/w). To make nanoemulsion, organic phase which constituted chia oil and Tween 80 as the surfactant were mixed. Then this mixture was stirred on magnetic stirrer at 750 rpm at 25^{o}C for 30 minutes. After mixing the organic phase, this phase was titrated for each minute into water phase on a magnetic stirrer at 700 rpm at 25^{o}C (Gulotta, Saberi, Nicoli and McClements, 2014).

2.2.3. Physical and Chemical Characterization of Chia Seed Oil and Emulsions

2.2.3.1. Antioxidant Activity

2.2.3.1.1. DPPH Radical Scavenging Method

In order to determine the antioxidant activity of both oil and emulsion samples, DPPH antioxidant measurement assay was used (Brand- Williams et.al., 1995). Firstly, extraction solvent (ES) was prepared by mixing 50 mL ethanol, 8 mL acetic acid and 42 mL of water. Then, for DPPH solution, 5 mg of DPPH reagent and 200 mL methanol were mixed. As the final stage, 0.1 g of each sample was mixed with the ES and DPPH reagent solution. Finalized solution was kept at dark at room temperature for 1 hour. Absorbance values were recorded at 517 nm using UV Spectrophotometer. (Optizen Pop Nano Bio, Korea) (Xuan, Gangqiang, Minh, Quy and Khanh, 2018). At the end of the experiment % radical scavenging activity was calculated by the following equation (Eq. 2.1).

$$\% \text{ DPPH Activity} = \frac{Abs\_control - Abs\_sample}{Abs\_control} x\ 100 \qquad \textbf{(Eq 2.3)}$$

-Abs control: the absorbance value of control group

-Abs sample: the absorbance value of control group oil/emulsion sample

2.2.3.1.2. Ferric Reducing Power of Antioxidant Activity (FRAP)

Since chia seed oil and its emulsion systems contain significant amount of ferric components (Gupta, 2015), in order to make cross check for antioxidant availability, FRAP assay was also used. FRAP assay is based on the redox reaction of iron containing complex compounds. Similar to DPPH radical scavenging method, spectrometric measurements was performed in this method (N., S., Rajurkar, 2011). The procedure is based on Benzie and Strain (1996) principle. Firstly, FRAP reagent was prepared. 0.3 M acetate buffer at a volume of 25 mL, TPTZ reagent (2,4,6-tris(2-pyridyl)-s-triazine) at a volume of 2.5 mL, TPTZ reagent of 10 mM in 40 mM HCl and 20 mM ferric chloride with the volume of 2.5 mL were mixed. This mixture was stored in dark and at room temperature. Then, 20μL sample was vigorously mixed with approximately 2 mL of FRAP reagent. The same procedure was followed for the Trolox standard solution. The mixture was kept in water bath at 37ºC for 30-40 minutes. Absorbance values were recorded at 593 nm by using a UV Spectrophotometer (Optizen Pop Nano Bio, Korea). At the same time, Trolox calibration curve data were prepared (Scapin, Schmidt, Prestes and Rosa, 2016).

2.2.3.2. Characterization of Total Phenolic Content (TPC)

For both oil and emulsion samples, total phenolic compound amount was determined by using Folin Ciocalteu total phenolic content method. Adjusted versions of the Folin Ciocalteu assay mentioned by Scapin, Schmidt, Prestes and Rosa (2016) was used. Minor revisions were done based on the type of the sample used in this study. Oil and emulsion samples were mixed with the mixture of ethanol, water and acetic acid at a ratio of 1:10 (v/v). Ethanol, acetic acid and water were seperately mixed in at 50:42:8

(v/v/v/) ratio. Sample and the chemicals were shaked in a shaker for a short time. This step was necessary in to release all the phenolic compounds from the samples. To achieve this, samples were kept in dark and at room conditions for 1 hour. After this first stage, mixtures were filtered using 0.45µm plastic recyclable micro-filters. Then, samples were mixed with the Folin Ciocalteu reagent. The second mixture obtained by mixing Folin reagent and sample were kept at dark room temperature for a short time. As the next phase, 7.5 % (w/v) ratio of $NaHCO_3$ was put to the kept mixture with a volume of approximately 1.5 mL. Finalized mixture was again held at dark for 45 minutes. Finally, absorbance value at 765 nm was read by a UV Spectrophotometer (Optizen Pop Nano Bio, Korea). Calibration curve was obtained by using gallic acid. All the data were recorded as gallic acid equivalent per amount of sample (Scapin, Schmidt, Prestes and Rosa, 2016).

2.2.3.3. Peroxide Values

To determine the oxidative stability of oil and emulsion samples, peroxide values were performed (Eq 2.2.). Procedure was applied according to International Fragrance Association Analytical Guidelines (2011). Firstly, 1 g of oil /emulsion samples were weighed in a flask 250 mL. Then 10 mL of chloroform was added to acetic acid and potassium iodide (KI) mixture which was obtained by mixing acetic acid and KI at ratio of 15: 1 (v/v) in total 50 mL. Then the total mixture was shaken vigorously. As the final step, 1 mL of potato starch solution and around 80 mL of water was added to the solution. Finally, titration was performed by 0.01/0.02 (depending on the PV value) $Na_2S_2O_3$ solution. Normality of the $Na_2S_2O_3$ solution was selected with respect to the number of peroxide value. If the *expected* peroxide value from the oil or emulsion sample was less than 12.5, then lower normality of $Na_2S_2O_3$ was used. Following equation was used to calculate the values.

$$\text{Peroxide value} = \frac{(Vf - Vi)x\ 1000xc}{m} \qquad \textbf{(Eq 2.4.)}$$

V_f= volume required of 0.1 N $Na_2S_2O_3$ for the oil or emulsion sample (mL)

V_i= volume required of 0.1 N $Na_2S_2O_3$ for the blank (mL)

c = molar concentration of $Na_2S_2O_3$ (M)

m = amount of sample used (g)

2.2.3.4. Determination of Thiobarbituric Acid Reactive Substances (TBARS)

For the secondary oxidation products determination, TBA assay was performed (Food TBARS Assay Kit by Oxford Biomedical Research, Inc (n.d.)). In this method, the reaction between 2- thiobarbituric acid assay and emulsion or oil samples was analyzed. 200 mg of samples were weighted and 1- butanol was added to a 25 mL of erlenmeyer flask. Then, 5 mL of this mixture and 5 mL of TBA reagent were prepared by putting 200 mg of thiobarbituric acid into 100 mL of 1- butanol using an ultrasound bath. As a next step, all mixture was kept in water bath at 95°C for 2 hours. After cooling the solution at room temperature, absorbance values at 530 nm were recorded by the help of UV Spectrophotometer. For emulsions, TBARS analysis was performed periodically, starting at the beginning and then at 4, 8 and 16 weeks. Emulsion samples were placed into 20 ± 3°C in laboratory condition. TBA value was calculated by the following equation.

$$\text{TBA value} = \frac{50\ x\ (X-Y)}{m} \qquad \textbf{(Eq 2.5.)}$$

X = absorbance value of sample at 530 nm

Y = absorbance value of blank at 530 nm

m= amount of sample, mg

50^* = used as a correlation constant if TBA reagent is prepared for 25 mL.

2.2.3.5. Mean Particle Size Determination

Mean particle size characterizations were performed using Malvern Mastersizer 2000n (Kineksus, UK) and Malvern Nano ZS90 in METU Central Laboratory. For the particle size measurements, refractive index was used in the range of 1.36-1.47 with the stirrer speed of 2,100-2,600 rpm. Then, D[3,2] Sauter mean particle size measurements were calculated. For each type of emulsion (0.5, 1, 1.25, 1.5, 2.5, 3 % oil concentrations (w/w)) approximately 16 trials were carried out. According to the results, emulsions were tried to be classified as nano.

2.2.3.6. Nuclear Magnetic Resonance Relaxometry

After obtaining the emulsion systems, to see the effect of high hydrostatic pressure, molecular interactions between oil and water phases and interpret the emulsion stability, Time Domain NMR relaxometry experiments were performed. Spin Track NMR system operating at a frequency of of 20.34 MHz (Resonance Systems GmbH, Germany) with an RF coil size of 10 mm was used to measure T_2 relaxation times. A CPMG pulse sequence at a repetition time of 3s, number of scans of 16, echo time of 4 ms, echo number of 2,500 were used.

2.2.3.7. Confocal Laser Scanning Microscopy

After observing the largest droplet sizes, to understand the mechanism of particle size growth after HHP, Confocal Laser Scanning Microscopy (CLSCM) at METU Molecular Biology and Biotechnology R&D Center (Zeiss LSM 880, UK) was used. 40X microscope objective was selected. Excitation parameters were set as 488/514/543 nm. Also, emission was performed at 560 nm. Images of different emulsions (0.5, 1, 1.25, 1.5, 2.5, 3 % oil concentrations (w/w)) were examined and particle size growth mechanisms were examined. No dye was used due to inherent fluorescence property of chia oil.

2.2.3.8. Instantaneous and Long-Term Physical Stability

Instantaneous stability of emulsions was also determined. Oil and water were mixed in 1.5/ 2 mL microtubes and initial height was recorded in centimeter. Then, 10,000 rpm for 1 min. was used for centrifugation at a high-speed mini centrifuge (MicroSpin12, USA). Final height (cm) was written as the phase separation from the oil and water mixture.

The ratio of the height of separated oil phase to the initial phase height was interpreted as the instantaneous stability of chia oil in water during emulsion preparation.

In addition, physical stabilities of emulsions were examined after 4 months that had been stored at the storage conditions of 20^{o}C and 50 % RH.

2.3. Experimental Design

Table 2.1 shows the important parameters for oil and emulsion samples.

Table 2.1. Representation of Factors and Levels of the Study

Experiments	**Factors**	**Levels / Conditions**
o Extraction Yield o Antioxidant Activity by FRAP Iron Reducing Method o Antioxidant Activity by DPPH Method o Peroxide Value o Secondary Oxidation Products by TBARS o Total Phenolic Content o Mean Particle Size o Oil- Water Interaction in Emulsion by Nuclear Magnetic Resonance Relaxometry (NMR) o Instanetous and Long Term Emulsion Stability	Oil Extraction Method Oil Concentration Emulsions Surfactant to Oil Ratio (SOR) (w/w) HHP Treatment High Hydrostatic Pressure (30 ^{o}C , 5 min)	Maceration Soxhlet Extraction HHP 0.5, 1, 1.25, 1.5, 2.5, 3 % (w/w) 1, 2, 3, 4 HHP / without HHP 300 and 500 MPa

2.4. Statistical Analysis

Analysis of Variance (ANOVA) was performed to find out the differences between samples using MINITAB (Version 16.1.0.0, Minitab Inc., Coventry, UK). Effect of HHP on oil yield and particle size of emulsions, effect of SOR and oil concentration on antioxidant activity, total phenolic content, peroxide value were all examined by Besides, correlation tests were applied for two different antioxidant activity measurement methods and for the NMR Relaxation times. Tukey's test was used as the multiple comparison test at a confidence level of 95% ($p = 0.05$). Results were obtained by using triple trials ($n=3$) for emulsions and eighteen trials for oil samples ($n= 18$).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Pretreatment for Chia Seed

It was concluded that chia seed samples should be grinded initially to increase the surface area for oil extraction. By applying this pretreatment, the efficiency of oil extraction could improve (Disher, Ali and Alhattab, 2015).

3.2. Oil Extraction from Chia Seed Samples

One of the the key aspects of this study is the extraction of oil from grinded chia seed samples. After obtaining oil from two different extraction methods, oil yields were calculated as the mean of 18 trials. Oil yield found in conventional extraction method was 29 %, which is higher compared to the yield of soxhlet extraction that was found as 25.97%.

According to the literature, chia oil is generally composed of 30-40 % carbohydrate, 20-25 % protein, 20-30 % dietary fiber, 28-33 % oil and some minerals and vitamins (Campos, Solís, Rubio, Guerrero and Ancona, 2014). The composition of oil extracted for this study was found in these ranges.

3.3. Effect of High Hydrostatic Pressure Treatment on Oil Yield

Another method applied for the oil extraction was HHP. To our knowledge, there is no study that investigated the effect of HHP on different plant seed sources. However, high hydrostatic pressure has a strong impact on cell membrane or wall structures of plant derived food systems (Bigikocin, Mert and Alpas, 2011). High pressure is also known to damage the cell wall so that active compounds from the cell structures can

be released. This is also explained as the enhancement of the bioavailability of targeted compounds. In a recent study, HHP was applied on onion for the purpose of obtaining certain compounds and it was found that pressure values more than 400 MPa were very effective for the extraction of valuable compounds (Vázquez-Gutiérrez, Carrión, Quiles, Puig and Hernando, 2012).

It was hypothesized in the study that oil extraction using high hydrostatic pressure would enhance the oil yield compared to solvent extraction. Results showed that oil yield increased from 29 % to 33 % for the same amount of grinded chia seed.

3.4. Chia Seed Oil Quality Parameters

3.4.1. Fatty Acid Composition of Chia Seed Oil

There are limited number of studies that examined the oil composition and functional characteristics of chia seed oil. In a study (Imran, Nadeem, Manzoor, Javed, Ali, Akhtar and Husain, 2016), fatty acid composition of oil extracted from Mexican chia seeds, was found to contain significant amount of polyunsaturated fatty acid, specifically ω-3 and ω-6 which were linolenic and linoleic acid groups. The composition was reported as 21.5 % linoleic and 55-57% linolenic acid (Campos, Solís, Rubio, Guerrero and Ancona, 2014). The analysis of oil extracted from black and white Australian chia seeds, showed a different fatty acid profile. Black and white chia seed oil samples were found to contain 2.97 % and 3.54 % linolenic (ω6) acid respectively. In addition, arachidic acid group, a fatty acid with 20 carbon without double bond, also had the highest proportion among these Australian origin chia oil samples (Noshe and Al-Bayyar, 2017). Researchers also showed that the Mexican origin seeds, that were cultivated at different months showed difference. Thus, temperature was found to be an important factor affecting fatty acid composition as well.

The origin of the chia seed, geographical conditions, soil type and color are all important parameters affecting the fatty acid profile of chia seed oil.

In this study, fatty acid composition was also determined. According to the results represented in **Table 3.1**, linolenic (ω-3) and linoleic (ω-6) fatty acid groups were found in the highest amount (62.85 % and 17.75 % respectively). These ratios were consistent with the previous studies. Palmitic and oleic acids were the other fatty acids found in significant amounts.

Table 3.1. Studied Chia Oil Fatty Acid Composition by GC

Fatty Acid % (w)	**Carbon number**	**Average %**
Palmitic	**16:00**	7.61
Stearic	**18:00**	3.5
Oleic	**18:01**	6.73
Linoleic (ω-6)	**18:02**	**17.75**
Linolenic (ω-3)	**18:03**	**62.85**
Other		1.56

3.4.2. Antioxidant Values by DPPH Radical Scavenging Method

Antioxidant activity of oil samples were also determined. Results had an average value of 14.16 ± 0.37 mg Trolox per g of oil. Antioxidant activity which were expressed as higher free radical scavenging ability was comparable with the results in other researches (Scapin, Schmidt, Prestes and Rosa, 2016). It was found that DPPH scavenging activity (%) was between 10.12 ± 0.79 and 32.35 ± 2.31 depending on the origin of chia seed (Gupta and Kaur, 2016). According to this, the results found in this study were consistent with the other studies.

3.4.3. Antioxidant Values by FRAP Iron Reduction Method

Antioxidant activity was also found using FRAP method. The results had an average value of 24.16 ± 0.55 mg Trolox/g ET oil sample. It was seen that FRAP iron reduction activity (%) was between 18.05 ± 3.10 and 45.04 ± 4.01 depending on the type of chia seed (Scapin, Schmidt, Preste and Rosa, 2016). Based on this, the results found in this study were found within this range as well.

3.4.4. Total Phenolic Content

Total phenolic content of oil samples was also measured and was found to have a value of 24.38 ± 0.80 mg GAE/ g oil sample. As predicted, chia oil which contained significant amount of antioxidant also comprised of high amount of total phenolic content. It was shown that total phenolic content was different from the literature values (Alves, Cazarin, Costa and Marostica, 2017). The difference in total phenolic content could be due to two factors; cultivation conditions and the methods used for extraction of phenolic compounds (Ayerza, 1995; Ayerza and Coates, 2004; Ayerza, 2010; Ayerza, 2013).

3.4.5. Peroxide Value

Peroxide value for oil samples was found as 4.66 ± 0.30 meq O_2 / g oil sample. It was found in a study that for South American origin chia seeds' peroxide values ranged between 2.7 to 3.8 meq O_2 / kg oil (Imran, Nadeem, Manzoor, Javed, Ali, Akhtar and Husain, 2016). Since the used chia seed was originated from South America in this study, the comparison was made among South America origin chia seed oil samples. Lower peroxide value can be interpreted as a good quality chia seed oil. Commercially sold vegetable oils were found to have peroxide values between 0.74 – 5.79 meq O_2 / g oil sample (Vidrih, Vidakovic and Abramovic, 2010). According to this value, it can be generalized that based on this range, chia oil was found between the peroxide value ranges for different vegetable oils.

3.4.6. Effect of HHP on Oil Yield

High hydrostatic pressure (300 MPa and 500 MPa) was applied to grinded chia seeds at 30^{o}C for 5 minutes. For both pressure values, oil yield increased from 29% (using conventional extraction method) to 33%. However, there was no significant difference between oil extraction yield at different pressure values ($p > 0.05$). Also, there was no significant effect of HHP on oil yield ($p > 0.05$). In addition to these, HHP did not affect the quality of the extracted chia oil.

3.5. Emulsion Quality Parameters

3.5.1. Mean Particle Size Measurements

When emulsions were prepared by spontaneous emulsification, the most vital point was analyzing the particle size of different emulsion systems. Since the stability, storage conditions, color and even functionality and chemical properties of emulsions were dependent on the range of particle size, mean particle size was significant.

De Sauter mean diameter (D[3,2]) was taken into consideration rather than volume mean diameter, D[4,3]. Mean particle size of emulsions with SOR of 1, 2, 2.5, 2.75, 3 and 4 are shown in the Table 3. It was found that up to a certain surfactant to oil ratio (SOR), emulsion system may be unstable since mean particle size is large. Therefore, finding the most suitable surfactant to oil ratio is important (Teng, Hu, Wang and Tao, 2017).

It was found that oil concentration had a significant effect on particle size of emulsions ($p < 0.05$). Furthermore, mean particle size of emulsions with oil concentrations of 1.25 %, 2.5 % and 3 % (w/w) were significantly different than the other samples ($p<0.05$).

Table 3. 2. De Sauter Mean Diameters of Emulsions Prepared with Different SOR

SOR (Surfactant to Oil Ratio)	D [3,2] (nm)
1	194 ± 0.06
2	179 ± 0.04
2.5	285 ± 0.37
2.75	121 ± 0.03
3	147 ± 0.003
4	90 ± 0.002

Up to SOR of 4 emulsions were not physically stable and phase seperation was observed immediately. Furthermore, SOR of 4 showed the lowest particle size. Thus, SOR of 4 was selected as the main ratio for emulsion preparation. All emulsions were prepared with SOR of 4 were nanoemulsions. The emulsions with surfactant to oil ratio of 4 also showed transparent image at the end of the storage period of 4 months. It was supported that using more than 3 times of surfactant in weight basis gave nano particle size for fish and lemon oil which were also prepared by spontaneous emulsification method (Gulatta, Saberi, Nicoli and McClements, 2014). This finding from the literature also promoted surfactant and oil ratio in this study.

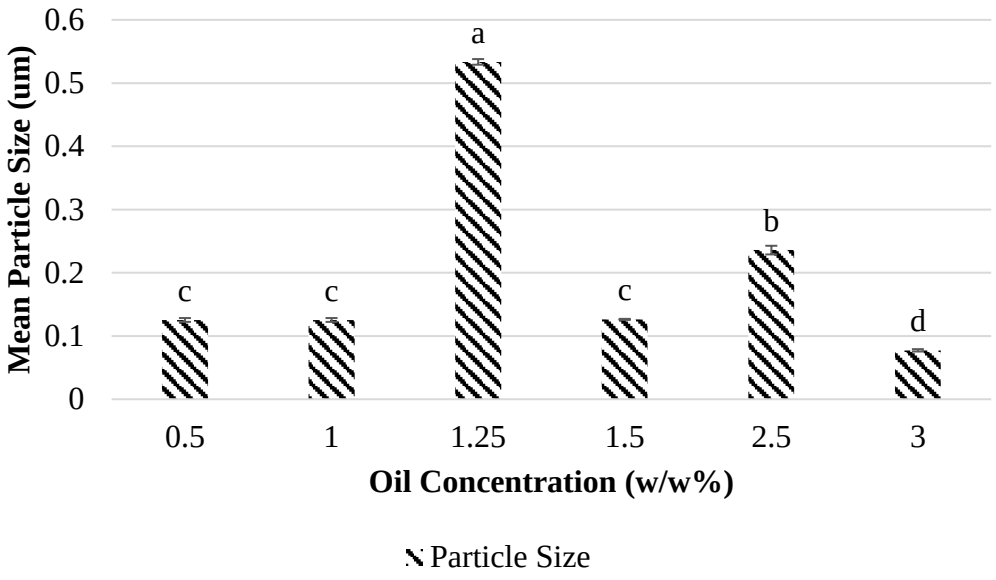

Figure 3.1. Particle Size of Different Emulsions

3.5.2. Effect of High Hydrostatic Pressure on Particle Size of Emulsions

HHP was also applied on emulsions at 2 different high-pressure values. However, pressure had a negative effect on the particle size and particle size values increased with pressure. It can be seen in **Table 3.3**. It was seen that the growth in particle size was significantly different for 300 MPa and 500 MPa ($p<0.05$). Normally, it might be expected that the higher the pressure, the larger the obtained particle size (Sevdin, Alpas and Yucel, 2017). However, the trend was different in this study. For oil concentrations other than 1.25% and 1.5%, the results show a higher mean particle size for emulsions treated with 500 MPa.

Table 3.3. Particle Size Before and After HHP Treatment

	Mean Particle Size (μm)		
Oil Concentration % (w/w)	Control Emulsions Prepared with 0.1 MPa (1 atm)	Emulsions treated with 300 MPa	Emulsions treated with 500 MPa
0.5	0.125 ± 0.03^{c}	35.3 ± 0.471^{a}	51.125 ± 1.113^{a}
1	0.125 ± 0.03^{c}	2.33 ± 0.285^{d}	2.892 ± 0.027^{b}
1.25	0.533 ± 0.04^{a}	3.264 ± 0.042^{c}	2.468 ± 0.050^{bc}
1.5	0.126 ± 0.01^{c}	5.131 ± 0.051^{b}	2.596 ± 0.008^{b}
2.5	0.236 ± 0.07^{b}	1.731 ± 0.021^{d}	3.35 ± 0.004^{b}
3	0.077 ± 0.02^{d}	0.549 ± 0.098^{e}	0.692 ± 0.107^{c}

In order to understand the probable mechanisms of particle growth, Confocal Laser Scanning Microscopy experiments were performed. With the help of the confocal microscopy, enhanced visuals of emulsions were obtained (**Figure 3.2 & 3.3**).

As it can be seen in the following figures, emulsions had multiple layers. For the samples without HHP application, single droplets within the emulsions can be seen in the **Figure 3.2**. Before HHP treatment, single droplets within the emulsion system were observed due to inherent fluorescence property of chia oil. Perfect sphere droplets were seen. On the other hand, when high pressure was applied, a more complex emulsion structure was formed as can be seen in **Figure 3.3**.

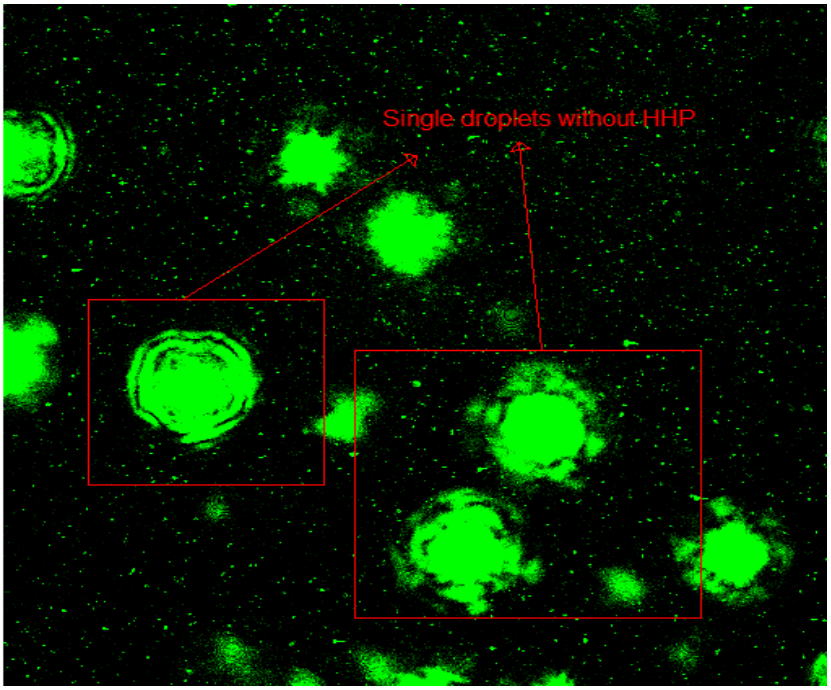

Figure 3.2. Illustration of Single Droplet Formed Without HHP

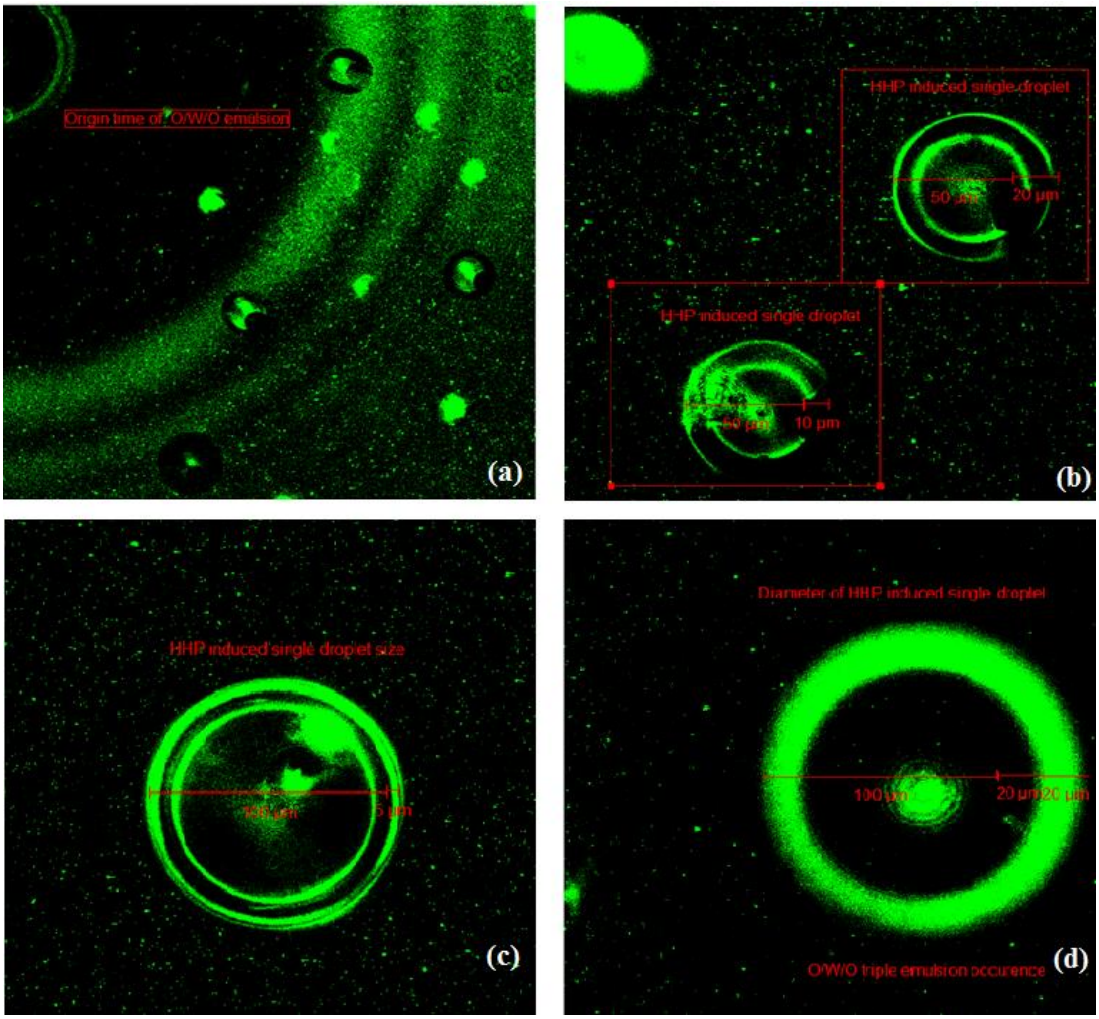

Figure 3.3. Illustrations of Emulsions Right After HHP Treatment

When 300 and 500 MPa pressures were applied at the temperature of 30°C for 5 minutes, droplets within the emulsion systems showed same behavior. Since the samples were placed into aqueous chamber, high pressure was transferred through water to the samples. By this way, water became compressible and conducted the high pressure to the droplets found in the emulsions. Thus, droplets were affected from external pressure. According to the images obtained by microscopy, owing to the

ability of Tween 80 as being resistant to pressure, it was not ruptured completely and instead, the surface of the droplet weakened. It was attached to both oil within the emulsion and oil found in the neighboring droplets. Thus, a new bigger droplet was formed and that was clearly seen in the microscopy images. This mechanism was explained by "coalescence". Coalescence is defined as formation of a single bigger droplet as a result of formational alteration of surfactant (Pena, 2003). **Figure 3.3a** denotes the immediate image of droplets after HHP treatment. At that stage, droplets started to come to each other and, firstly, flocculated. After 4 hours, they created new bigger droplet due to complete coalescence mechanism. Microscopic image of multiple O/W/O droplets can be seen in **Figure 3.3b, c** and **d**.

3.5.3. Stability of Emulsions

3.5.3.1. Characterization of Long-Term Stability

When emulsions were examined closely, it was seen that, there was no significant phase separation was observed for 4 months. As a result, it was concluded that, emulsions had good stability.

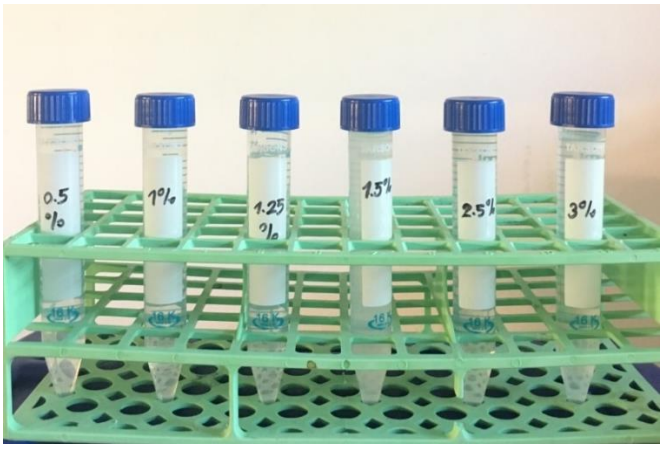

Figure 3.4. Illustration of Six Different Concentrated Emulsions

3.5.3.2. Characterization of Instantaneous Stability

According to the results of 18 emulsion trials, instantaneous stability of emulsion systems had an average value between 97-98%. This numeric evaluation explained the strong chemical harmony between surfactant, oil and water phases in the ratio of 97-98% (Demiralp and Tunga, 2015).

3.5.3.3. Characterization of Stability by NMR Relaxometry

According to the experimental results, it was seen that as the oil concentration increased, T_2 values decreased due to less free mobile water molecules. Average T_2 values can be seen in **Table 3.3**. A large negative correlation ($r=0.803$) between T_2 values and oil concentration was found ($p<0.05$).

HHP had an anticipated effect on T_2 values of emulsions. This effect was explained by the growth in particle sizes. Enlargement in particle size led to a decrease in the surface area. Therefore, fewer water molecules were attached to the surfactant. For emulsion systems, particle size changes were only measurable by NMR if coalescence mechanism took place during instability. This explained the signals perceived by NMR Relaxometry device since HHP-caused enlargement mechanism was also explained with coalescence formation in this study. As a result of this, bigger droplets were found freely within the new pressurized dispersion system and due to this, free hydrogen molecules coming from water were higher due to a lower surface area. After HHP application, higher free mobile water resulted in longer T_2 value (Rismanto and Zwaag, 2007).

When the statistical analysis for HHP process and oil concentrations were investigated, it was seen that both the presence of HHP treatment and oil concentration within the emulsion had significant effects on T_2 values separately ($p < 0.05$). On the other hand, their interactions were not significant on T_2 value of emulsions ($p > 0.05$).

Table 3.4. Average T_2 Values of Emulsions

Oil Concentration of Samples % (w/w)	Average T_2 Values (ms)
0.5	1920.91 ± 109.75^{a}
1	1777.85 ± 26.21^{ab}
1.25	1696.35 ± 82.96^{ab}
1.5	1787.04 ± 127.71^{ab}
2.5	1501.89 ± 110.74^{ab}
3	1356.38 ± 37.24^{b}

Table 3.5. Average T_2 Values of HHP Treated Emulsions

Oil Concentration of Samples % (w/w)	HHP Induced Average T_2 Values (ms)
0.5	2806.20 ± 50.63^{a}
1	2670.59 ± 25.81^{ab}
1.25	2566.57 ± 21.42^{b}
1.5	2544.14 ± 23.61^{b}
2.5	2395.13 ± 21.59^{c}
3	2059.01 ± 21.99^{d}

3.5.4. Antioxidant Activity of Emulsions by DPPH Radical Scavenging Method

Since the amount of unsaturated fatty acids are quite high, chia oil itself does not have a high oxidative stability. However, emulsion systems were expected to decrease oxidation by embedding active antioxidant compounds inside of them. The % DPPH free radical scavenging activity of emulsions are shown in **Figure 3.5.** An analysis of

chia oil emulsions showed that they belong to medium oxidative stable oil groups (Bodoira, Penci, Ribotta and Martinez, 2017). When compared (**Figure 3.6**)**,** it was seen that antioxidant activity of emulsions were higher than oil samples. There was significant effect of oil concentration on DPPH free radical scavenging antioxidant activity ($p < 0.05$).

The reason for an increase in the antioxidant activity as oil concentration increased was explained by "polarity paradox of antioxidants" (Kiralan, Baykut, Kittipongpittaya, McClements and Decker, 2014). In chia oil, most of the antioxidative compounds have non-polar structures. It was proven that these compounds showed better functionality in O/W emulsion systems rather than in oil form separately. Since antioxidants found in chia oil had non-polar property, they could bind to oil and stay there more effectively (Kiralan et. al., 2014). It could be concluded that emulsion systems preserved the active compounds which were normally very susceptible to oxidation. This hypothesis has been further supported in a study which was related to investigation of antioxidant activity in α, γ and δ tocopherol co-micelles. Similarly, in this study it was seen that active compounds were preserved more effectively within the micelle structures. (Kiralan, Baykut, Kittipongpittaya, McClements and Decker, 2014).

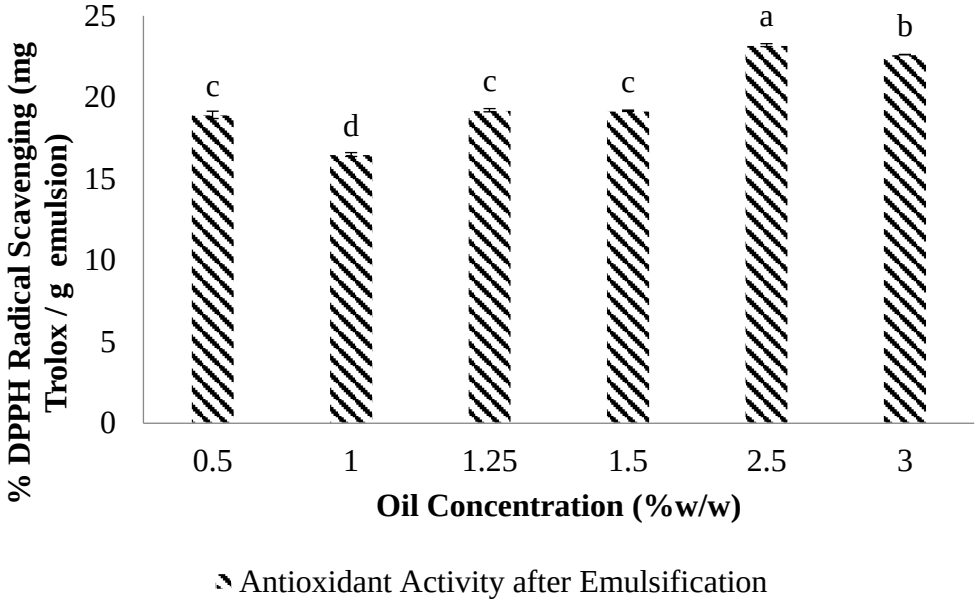

Figure 3.5. % DPPH Radical Scavenging Activity of Emulsions

Among the oil concentrations, emulsions with oil concentration of 1, 2.5 and 3 % (w/w) were significantly different compared to the others. Most importantly, emulsion with the oil concentration of 2.5 % (w/w) showed the highest antioxidant activity.

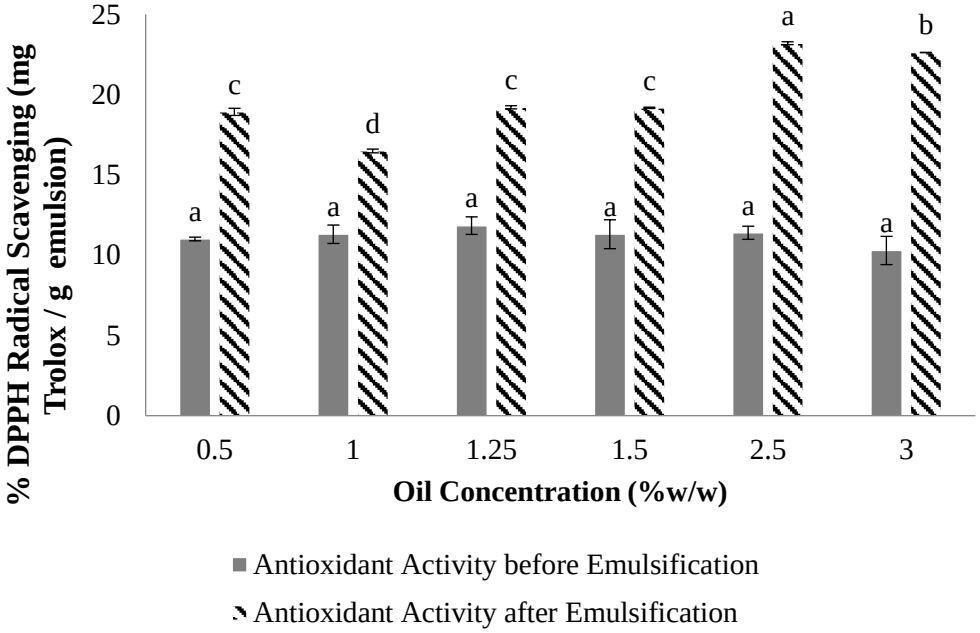

Figure 3.6. Effect of Antioxidant Activity by DPPH Method After Emulsification

3.5.5. Antioxidant Activity of Emulsions by FRAP Ferric Reducing Method

Antioxidant activity found by using FRAP method for emulsions was significantly higher compared to oil samples as in the case of DPPH method (Kiralan, Baykut, Kittipongpittaya, McClements and Decker, 2014). According to statistical results, there was significant effect of oil concentration on antioxidant activity by FRAP iron reduction method ($p < 0.05$). Moreover, like DPPH free radical scavenging method, 1, 2.5 and 3 % (w/w) oil containing emulsion systems were significantly different from each other.

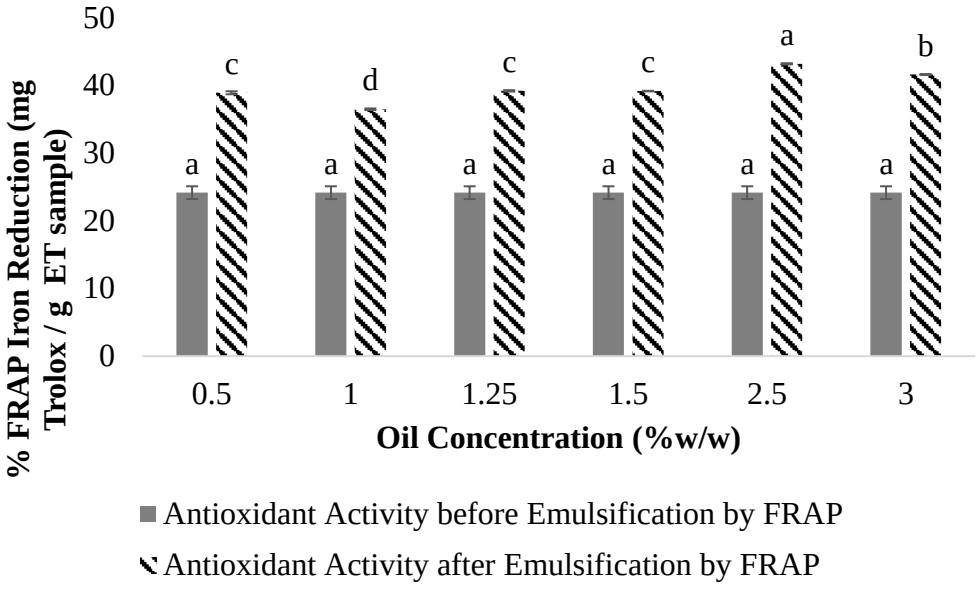

Figure 3.7. Effect of Antioxidant Activity by FRAP Method After Emulsification

3.5.6. Comparison and Correlation Between Antioxidant Activity Determination Methods of DPPH and FRAP

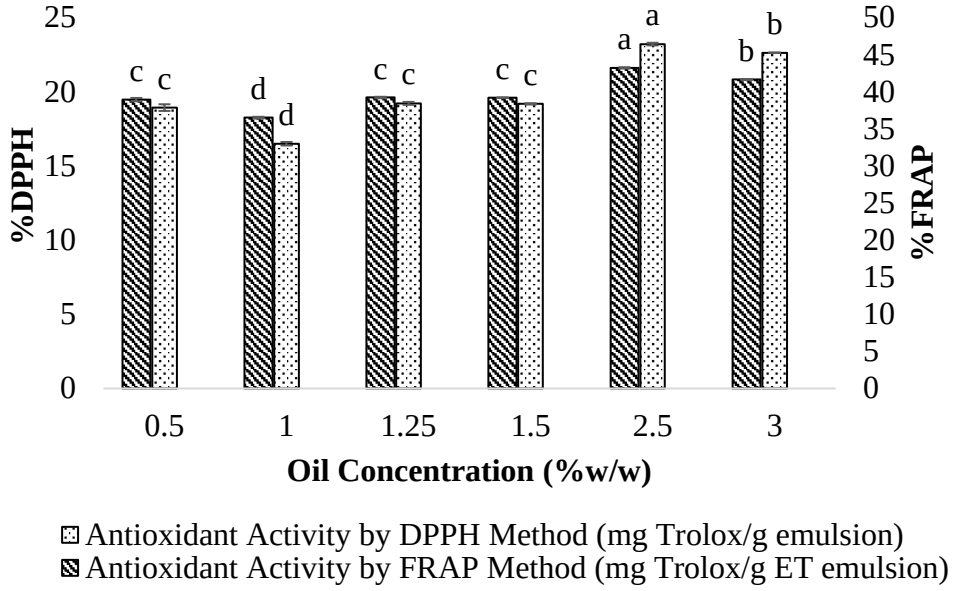

Figure 3.8. Comparison between DPPH and FRAP Antioxidant Methods

DPPH and FRAP methods were also compared. However, it is important to note that chemical mechanisms of FRAP and DPPH methods are different. For FRAP, reduction reaction of ferric to ferrous ion takes place and different compounds color change is important (Benzie & Strain, 1996). On the other hand, for DPPH method, electron transfer and different colored complex compounds are of concern. For these reasons, exact matching of quantitative data or even change for each oil concentration was not the case. When **Figure 3.9** was examined, it was obvious that the rate of change of antioxidant activities might be different for DPPH and FRAP. For instance, for emulsion with the oil concentration of 1%, free radical scavenging activity in terms of DPPH was higher than ferric reducing antioxidant activity in terms of FRAP. These

types of variations may be related to diversity of antioxidant compounds which acted for a specific assay (Ahoua, Kone, Konan, Trab and Bonfoh, 2012).

Overall, when a comparison is made (**Figure 3.9**), it was certain that, emulsion with oil concentration of 2.5 % (w/w) showed the greatest antioxidant activity for both methods. According to ANOVA result, there was strong positive correlation between DPPH and FRAP methods (r = 0.989). Also, this correlation was accepted as significant (p = 0.000).

3.5.7. Interpretation of Peroxide Value of Emulsions

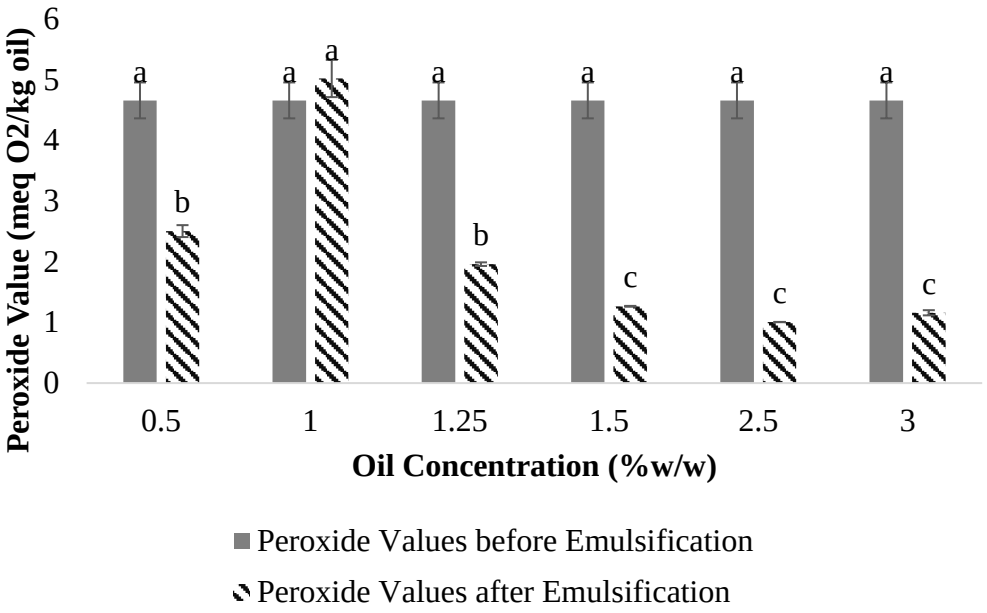

Figure 3.9. Identification of Peroxide Values before and after Emulsification

Detection of peroxide value was important since the formation of hydroperoxides as primary oxidation products was a crucial indicator for oxidative stability (Osborn and

Akoh, 2004). From **Figure 3.9,** it was seen that peroxide values of emulsions were lower than bulk oil samples. This can be explained as protection effect of emulsion droplets. Oil was embedded within a close system which retarded oxidation. Furthermore, degree of peroxide values was different for each emulsion samples. According to the statistical findings, there was a significant effect of oil concentration on peroxide values. ($p < 0.05$). Also, as it can be deduced from the **Figure 3.9** that peroxide value of emulsion with 1% (w/w) oil concentration was significantly higher from the other samples ($p<0.05$).

There are several reasons underlying the differences in peroxide values. There was an increasing, decreasing and again increasing trend between peroxide values of emulsions as the concentration of oil increased. According to a study, particle size of emulsions and their peroxide values are interrelated. As the particle size decreases, surface area increases within the continuous phase. This leads to an increase in oxidation since contact area with oxygen may be higher (Baysan, Yıldırım, Takma and Koç, 2018). Therefore, an increase in peroxide values for emulsions with 1 % and 3 % (w/w) oil may be linked to a decrease in their particle size (Baysan, Yıldırım, Takma and Koç, 2018).

According to the **Figure 3.9**, 2.5 % (w/w) oil containing emulsion was seen to have the lowest peroxide value since its antioxidant activity was in highest amount.

In addition, there was strong negative correlations between Peroxide and DPPH ($r = -0.815$) as well as Peroxide and FRAP values ($r= -0.824$) respectively. Furthermore, these correlations were significant ($p = 0.000$).

3.5.8. Thiobarbituric Acid Reactive Substances (TBARS) Analysis and Its Importance for Emulsion Systems

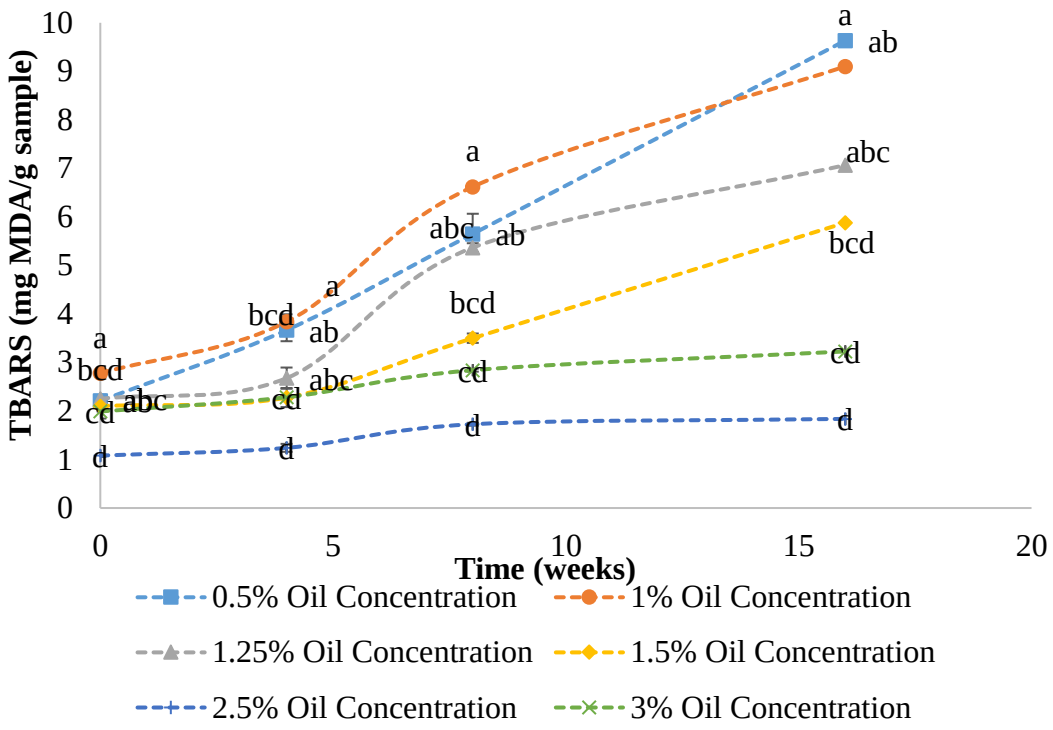

Figure 3.10. TBARS Values of Different Emulsions

As can be seen from **Figure 3.10**, there was further occurrence of oxidation. The number of compounds which turned from primary peroxide form into secondary form (aldehyde and ketones specifically) increased over time. There was a significant effect of oil concentration on TBARS values ($p < 0.05$).

Emulsion with 2.5% (w/w) formed secondary oxidation products in the least amount compared to other samples. This may be again explained by having higher antioxidant activity.

Moreover, there was strong negative correlations between TBARS and DPPH values (r = -0.841) as well as TBARS and FRAP values (r = -0.875) respectively. These correlations were found significant (p = 0.000).

3.5.9. Comparison of Peroxide Value and TBARS In Terms of Oxidative Stability

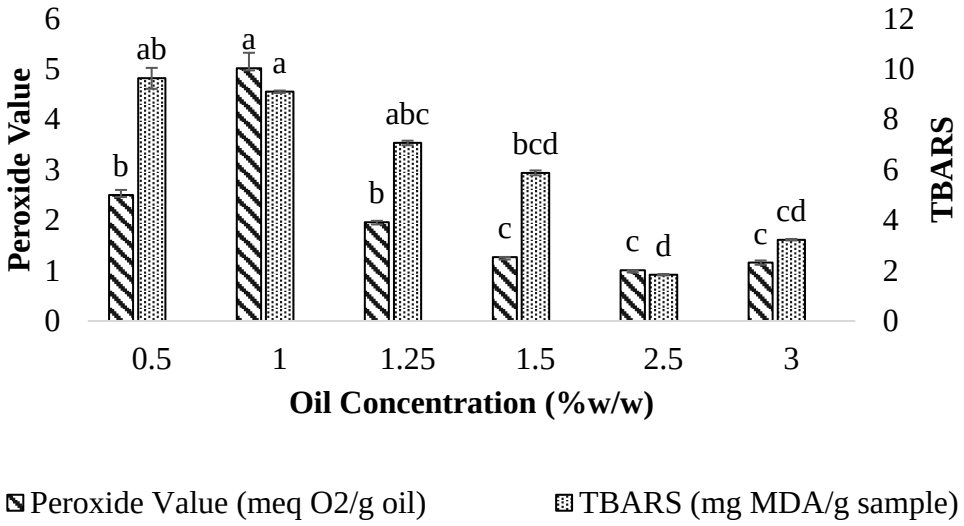

Figure 3.11. Comparison of Peroxide Value and TBARS of Emulsions

Correlation between peroxide and TBARS values was calculated to compare them. It was found that for peroxide value and TBARS data, correlation coefficient of r was 0.743, 0.813, 0.831 and 0.730 for 0, 4, 8 and 16 weeks respectively. They showed high positive correlation between them. Furthermore, these correlation values were statistically significant. ($p < 0.05$).

3.5.10. Comparison of Total Phenolic Content and Effect of Emulsions on Total Phenolic Content Analysis

Total phenolic content was found to be directly proportional to antioxidant activity of the samples. Important bioactive structures of flavonoids, phenolic compounds, phenolic acid groups were examined as the components of total phenolic content Most of these compounds contributed to antioxidant property (Chi-Tang Ho, 1992). For this reason, it was reasonable to obtain similar trend for antioxidant activity and total phenolic content. However, since both methods used different chemical techniques, it was not expected to see similar rate of change and numerical data between TPC and antioxidant result. There was a significant effect of oil concentration on total phenolic content ($p < 0.05$). It was also seen that the emulsions with the oil concentration of 1% (w/w) were different from the rest. This was related to less amount of antioxidant compounds.

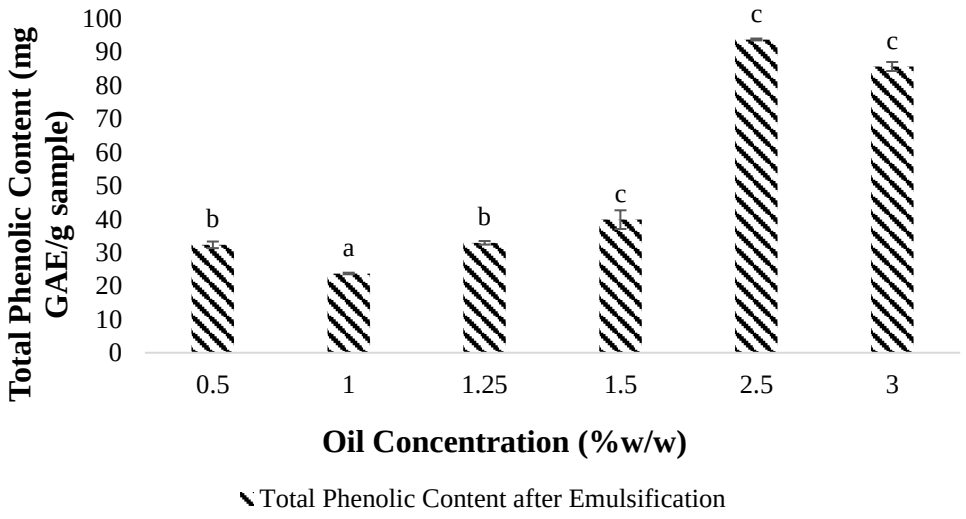

Figure 3.12. Total Phenolic Content of Different Emulsions

There was an increase in total phenolic content for emulsion systems compared to pure oil. Similarly, since most of the phenolic compounds had nonpolar features for chia oil, dispersion system provided a better activation environment. **Figure 3.13** shows the increase in total phenolic content after emulsification.

In addition to this, there was strong positive correlations between TPC and DPPH values (r = 0.957) as well as TPC and FRAP values (r = 0.936) respectively. Also, strong negative correlation was found between TPC and TBARS values (r = -0.809). These correlations were accepted as significant (p = 0.000 for each correlation).

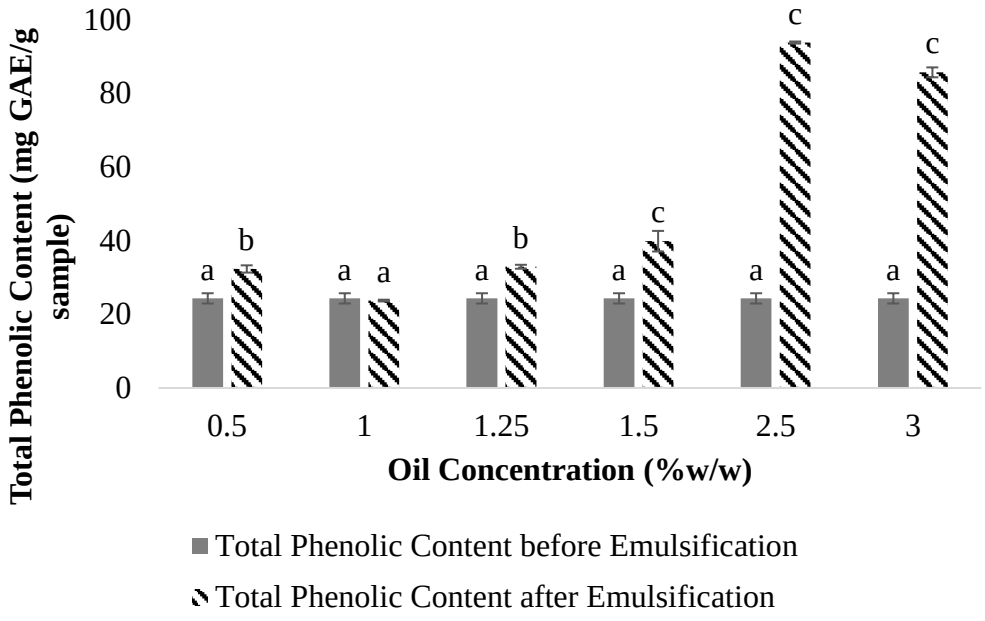

Figure 3.13. Comparison of Total Phenolic Content before and after Emulsification

According to particle size results, it was seen that the particle size of 2.5 % (w/w) oil containing emulsion system was not the smallest one, but it showed the best antioxidant activity and oxidative stability. Therefore, it cannot be concluded that, emulsions with lowest particle size had the most desired properties. It was deduced that when the amount of surfactant used was increased, some of the valuable compounds which have antioxidant or phenolic properties might be transferred to the continuous water phase with the excess amount of surfactant. As a result, carried compounds were insignificant in amount within the droplets. In other words, they were counted as affectless for chemical analyses performed for emulsions (Kiralan, Baykut, Kittipongpittaya, McClements and Decker, 2014). Thus, it was concluded that entrapping active compounds and increasing antioxidant availability as well as most obtaining high oxidative stability were more important rather than obtaining the smallest particle size.

In addition to this, strong correlations were found among all five different chemical analyses conducted for emulsion samples. Correlation coefficient were reported as **Appendice B.10**.

CHAPTER 4

CONCLUSION

Chia seed and its omega 3 and 6 rich oil components have become one of the attractive products recently in all over the world. Unfortunately, there are still few studies based on chia oil.

In this study chia oil extraction and physicochemical properties of the extracted oil were investigated. Soxhlet, maceration and HHP assisted solvent extraction were explored to find the highest yield from the seeds. Fatty acid composition, antioxidant activity by 2 methods (DPPH and FRAP), total phenolic compounds by Folin Ciocalteu method, peroxide value and TBARS experiments were performed on the extracted oil. In addition, to protect the nutritive value of the oil, nanoemulsions were prepared using spontaneous emulsification method. Tween 80 was used as the surfactant and surfactant to oil ratio of 4 was selected after preliminary experiments.

Effect of different oil ratios (0.5, 1, 1.25, 1.5, 2.5, 3 % (w/w)) on mean particle size, antioxidant activity, primary and secondary oxidation products of emulsions were also investigated. NMR Relaxometry by measuring T_2 relaxation times, stability experiments (instantaneous and long term) and confocal laser scanning microscopy experiments were also conducted on the emulsions.

It found that emulsions with oil concentration for 2.5 % (w/w) was the best choice in terms of antioxidant activity, total phenolic content, lowest peroxide and TBARS values and pretty good stability results. Furthermore, the amount of surfactant used for this emulsion system (10 % (w/w)) was within the limits of surfactant

concentrations for nanoemulsion systems. Unexpectedly, emulsions with higher oil concentration (3% (w/w)) did not give better qualitative and quantitative results.

One of the most novel technique that was applied in this study was the use of Spontaneous Emulsification, NMR Relaxometry and High Hydrostatic Pressure techniques on chia oil emulsion system. Since there is no literature study on chia seed oil nanoemulsions by using these approaches, this study was vital. This study proved that chia oil nanoemulsions showing antioxidant properties can be obtained by using spontaneous low energy method.

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APPENDICES

A. CALIBRATION CURVE

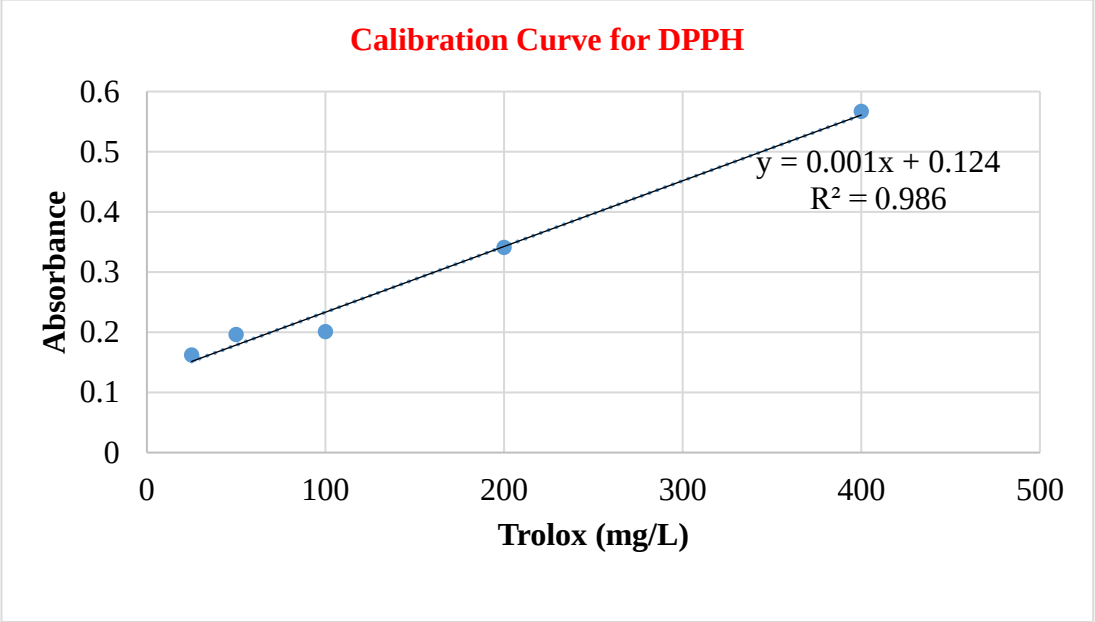

Figure A.1. Calibration Curve for DPPH

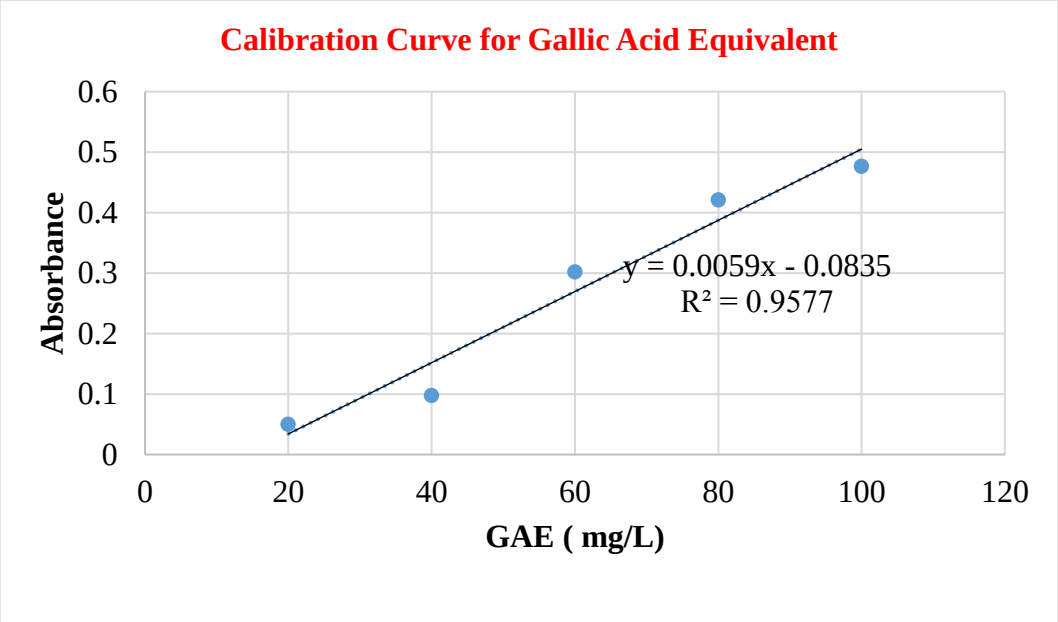

Figure A.2. Calibration Curve for Gallic Acid

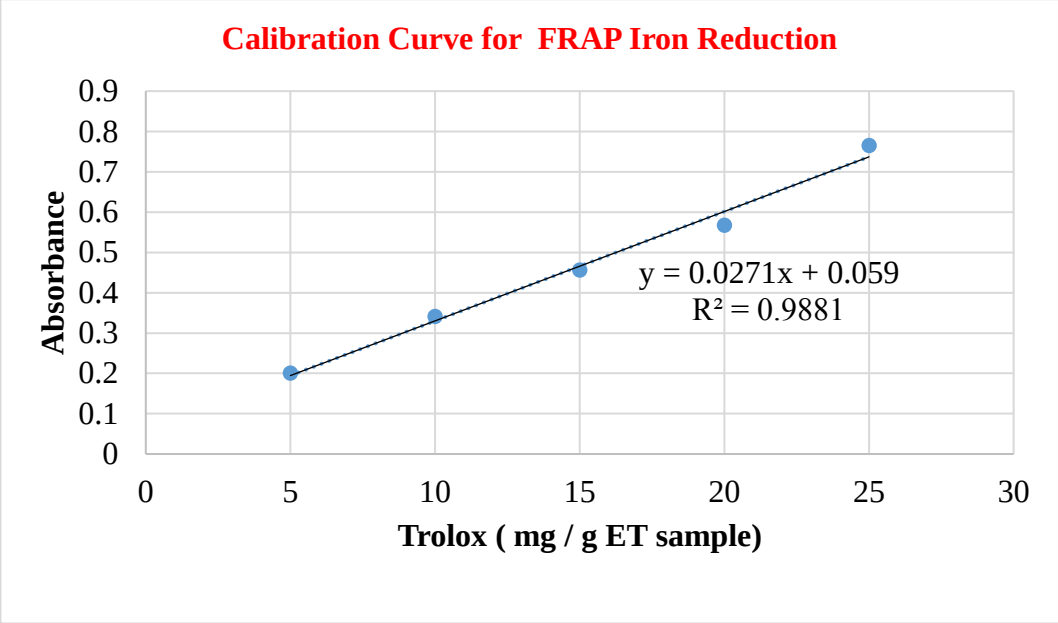

Figure A.3. Calibration Curve for FRAP Iron Reduction

B. STATISTICAL ANALYSES

Table B. 1. ANOVA and Tukey's Comparison Test with 95% confidence level for determining antioxidant activity of emulsions and oil samples with DPPH Method

Effect of Oil Concentrations within Emulsions on % DPPH Antioxidant Activity

Source	DF	SS	MS	F	P
Oil Samples	5	0,78	0,16	0,13	0,982
Error	12	14,36	1,20		
Total	17	15,14			

S = 1,094 R-Sq = 5,15% R-Sq(adj) = 0,00%

Grouping Information Using Tukey Method

Oil Samples	N	Mean	Grouping
1.5	3	14,509	A
0.5	3	14,258	A
1.25	3	14,209	A
1	3	14,157	A
3	3	13,956	A
2.5	3	13,869	A

One-way ANOVA: % DPPH Antioxidant Activity versus Oil Concentrations

Source	DF	SS	MS	F	P
Oil Concentrations	5	95,6641	19,1328	456,96	0,000
Error	12	0,5024	0,0419		
Total	17	96,1665			

S = 0,2046 R-Sq = 99,48% R-Sq(adj) = 99,26%

```
Grouping Information Using Tukey Method

Oil
Concentrations  N     Mean  Grouping
2,5             3  23,2025  A
3               3  22,6223    B
1,25            3  19,2093      C
1,5             3  19,1752      C
0,5             3  18,9249      C
1               3  16,4903        D

Means that do not share a letter are significantly different.
```

Table B. 2. ANOVA and Tukey's Comparison Test with 95% confidence level for determining antioxidant activity of oil samples and emulsions with FRAP Method.

One-way ANOVA: % FRAP Concentrations versus Oil Samples

```
Source       DF     SS    MS     F      P
Oil Samples   5   0,78  0,16  0,13  0,982
Error        12  14,36  1,20
Total        17  15,14

S = 1,094   R-Sq = 5,15%   R-Sq(adj) = 0,00%


Grouping Information Using Tukey Method

Oil
Samples  N   Mean  Grouping
1.5      3  24,509  A
0.5      3  24,258  A
1.25     3  24,209  A
1        3  24,157  A
3        3  23,956  A
2.5      3  23,869  A
```

One-way ANOVA: % FRAP Concentrations versus Oil Concentrations

```
Grouping Information Using Tukey Method
Oil
Concentrations  N     Mean  Grouping
2,50            3  43,2025  A
3,00            3  41,6223    B
1,25            3  39,2093      C
1,50            3  39,1752      C
0,50            3  38,9249      C
1,00            3  36,4903        D
```

Table B. 3. ANOVA and Tukey's Comparison Test with 95% confidence level for determining peroxide value of oil samples and emulsions

Effect of Oil Samples Extracted at Different Times on Peroxide Value Determination

One-way ANOVA: Peroxide Values versus Oil Samples

```
Source       DF      SS     MS     F      P
Oil Samples   5  14,937  2,987  3,03  0,054
Error        12  11,830  0,986
Total        17  26,767

S = 0,9929   R-Sq = 55,80%   R-Sq(adj) = 37,39%


Grouping Information Using Tukey Method

Oil
Samples  N    Mean  Grouping
6        3  5,5533  A
5        3  5,4167  A
4        3  5,2533  A
2        3  4,7667  A
1        3  3,9333  A
3        3  3,0117  A
```

One-way ANOVA: Peroxide Values versus Oil Concentrations

```
Source               DF       SS      MS       F      P
Oil Concentrations    5  34,3697  6,8739  129,07  0,000
Error                12   0,6391  0,0533
Total                17  35,0088

S = 0,2308   R-Sq = 98,17%   R-Sq(adj) = 97,41%

Grouping Information Using Tukey Method

Oil
Concentrations  N    Mean  Grouping
1,00            3  5,0167  A
0,50            3  2,5033    B
1,25            3  1,9590    B
1,50            3  1,2647      C
3,00            3  1,1583      C
2,50            3  1,0053      C


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Oil Concentrations
```

Table B. 4. ANOVA and Tukey's Comparison Test with 95% confidence level for determining time dependent peroxide values for emulsions

Correlations: Peroxide values; 0 weeks TBARS

```
Pearson correlation of Peroxide values and 0 weeks TBARS = 0,743
P-Value = 0,000
```

Correlations: Peroxide values; 4 weeks TBARS

```
Pearson correlation of Peroxide values and 4 weeks TBARS = 0,813
P-Value = 0,000
```

Correlations: Peroxide values; 8 WEEK TBARS

```
Pearson correlation of Peroxide values and 8 WEEK TBARS = 0,831
P-Value = 0,000
```

Correlations: Peroxide values; 16 WEEK TBARS

```
Pearson correlation of Peroxide values and 16 WEEK TBARS = 0,730
P-Value = 0,001
```

Effect of Oil Concentrations within Emulsions on TBARS

One-way ANOVA: TBARS versus Oil Concentrations

Source	DF	SS	MS	F	P
Oil Concentrations	5	173,44	34,69	11,03	0,000
Error	48	150,98	3,15		
Total	53	324,42			

S = 1,774 R-Sq = 53,46% R-Sq(adj) = 48,61%

Grouping Information Using Tukey Method

Oil Concentrations	N	Mean	Grouping
1,00	9	6,519	A
0,50	9	6,317	A B
1,25	9	5,037	A B C
1,50	9	3,886	B C D
3,00	9	2,783	C D
2,50	9	1,601	D

One-way ANOVA: TBARS versus time

Source	DF	SS	MS	F	P
time	3	178,22	59,41	18,26	0,000
Error	68	221,24	3,25		
Total	71	399,46			

S = 1,804 R-Sq = 44,62% R-Sq(adj) = 42,17%

Grouping Information Using Tukey Method

time	N	Mean	Grouping
16	18	6,124	A
8	18	4,284	B
4	18	2,663	C
0	18	2,075	C

General Linear Model: TBARS versus Oil concentration; time

```
Source              DF        SS       MS        F      P
Oil Concentration    5  3601,575  720,315  4725,05  0,000
Error               18     2,744    0,152
Total               23  3604,319

S = 0,3904   R-Sq = 99,92%   R-Sq(adj) = 99,90%


Grouping Information Using Tukey Method

Oil
Concentration  N    Mean  Grouping
0,50           4  35,300  A
1,50           4   5,230    B
1,25           4   3,263      C
1,00           4   2,330        D
2,50           4   1,737        D
3,00           4   0,674          E
```

Table B. 5. ANOVA and Tukey's Comparison Test with 95% confidence level for determining TPC of oil samples and emulsions

Effect of Oil Samples Extracted at Different Times on Total Phenolic Content

One-way ANOVA: TPC Concentrations versus Oil Samples

```
Source       DF     SS    MS     F      P
Oil Samples   5   8,02  1,60  0,77  0,587
Error        12  24,92  2,08
Total        17  32,94

S = 1,441   R-Sq = 24,35%   R-Sq(adj) = 0,00%


Grouping Information Using Tukey Method

Oil
Samples  N    Mean  Grouping
6        3  24,956  A
1        3  24,925  A
3        3  24,876  A
5        3  24,203  A
4        3  23,842  A
2        3  23,157  A
```

One-way ANOVA: TPC Concentrations versus Oil Concentrations %

Source	DF	SS	MS	F	P
Oil Samples	5	34,3697	6,8739	129,07	0,000
Error	12	0,6391	0,0533		
Total	17	35,0088			

S = 0,2308 R-Sq = 98,17% R-Sq(adj) = 97,41%

Grouping Information Using Tukey Method

Oil Samples	N	Mean	Grouping
1,00	3	5,0167	A
0,50	3	2,5033	B
1,25	3	1,9590	B
1,50	3	1,2647	C
3,00	3	1,1583	C
2,50	3	1,0053	C

Table B. 6. ANOVA and Tukey's Comparison Test with 95% confidence level for determining HHP effect on oil samples and emulsions

One-way ANOVA: Oil Yield Before HHP versus Oil Samples

Source	DF	SS	MS	F	P
Oil Samples	5	0,06045	0,01209	1,87	0,173
Error	12	0,07750	0,00646		
Total	17	0,13795			

S = 0,08036 R-Sq = 43,82% R-Sq(adj) = 20,41%

Grouping Information Using Tukey Method

Oil Samples	N	Mean	Grouping
6,00	3	2,97633	A
5,00	3	2,94667	A
1,00	3	2,93000	A
2,00	3	2,87167	A
4,00	3	2,84333	A
3,00	3	2,81467	A

One-way ANOVA: Oil Yield After HHP versus Oil Samples

```
Source       DF      SS      MS     F      P
Oil Samples   5  0,4447  0,0889  4,57  0,014
Error        12  0,2334  0,0194
Total        17  0,6781

S = 0,1395   R-Sq = 65,58%   R-Sq(adj) = 51,24%



Grouping Information Using Tukey Method

Oil
Samples  N    Mean  Grouping
6,00     3  3,2970  A
1,00     3  3,2333  A
5,00     3  3,0733  A B
2,00     3  3,0667  A B
4,00     3  3,0040  A B
3,00     3  2,8123    B
```

Table B. 7. ANOVA and Tukey's Comparison Test with 95% confidence level for determining particle size for different SOR

One-way ANOVA: D [3,2] Particle Size versus SOR

```
Source  DF       SS       MS       F      P
SOR      5  58,2027  11,6405  165,91  0,000
Error   90   6,3146   0,0702
Total   95  64,5173

S = 0,2649   R-Sq = 90,21%   R-Sq(adj) = 89,67%

Grouping Information Using Tukey Method


SOR    N    Mean  Grouping
2,50  16  2,6875  A
1,00  16  1,8569    B
3,00  16  1,4475      C
2,00  16  1,4450      C
2,75  16  1,2194      C
4,00  16  0,0761        D
```

Table B. 8. ANOVA and Tukey's Comparison Test with 95% confidence level for determining particle size for emulsion

Correlation

```
Pearson correlation of Oil Concentration and T2 Values = -0,803
P-Value = 0,000
```

One-way ANOVA: D [3,2] versus Oil Concentration

```
Source              DF        SS        MS         F      P
Oil Concentration    5  1,727854  0,345571  10383,16  0,000
Error              121  0,004027  0,000033
Total              126  1,731881

S = 0,005769   R-Sq = 99,77%   R-Sq(adj) = 99,76%
```

```
Grouping Information Using Tukey Method

Oil
Concentration   N     Mean  Grouping
1,25%          10  0,53347  A
2,50%          13  0,23587    B
1,50%          24  0,12629      C
1,00%          28  0,12539      C
0,50%          28  0,12539      C
3,00%          24  0,07737        D
```

One-way ANOVA: D[3,2] versus Oil Concentration after HHP

```
Source               DF        SS        MS        F      P
Oil Concentration %   5  7947,572  1589,514  2553,16  0,000
Error                18    11,206     0,623
Total                23  7958,778

S = 0,7890   R-Sq = 99,86%   R-Sq(adj) = 99,82%
```

```
Grouping Information Using Tukey Method

Oil
Concentration %  N    Mean  Grouping
0,50             4  51,175  A
2,50             4   3,350    B
1,00             4   2,860    B
1,50             4   2,595    B
1,25             4   2,422    B C
3,00             4   0,742      C
```

Table B. 9. ANOVA and Tukey's Comparison Test with 95% confidence level for determining T_2

Two-way ANOVA: Average T2 Values versus Process; Oil Concentration

Source	DF	SS	MS	F	P
Process	1	6253025	6253025	456,70	0,000
Oil Concentration	5	1598724	319745	23,35	0,000
Interaction	5	51112	10222	0,75	0,596
Error	24	328603	13692		
Total	35	8231465			

S = 117,0 R-Sq = 96,01% R-Sq(adj) = 94,18%

Two-way ANOVA: Average T2 Values versus Process; Oil Concentration

Source	DF	SS	MS	F	P
Process	1	6253025	6253025	456,70	0,000
Oil Concentration	5	1598724	319745	23,35	0,000
Interaction	5	51112	10222	0,75	0,596
Error	24	328603	13692		
Total	35	8231465			

S = 117,0 R-Sq = 96,01% R-Sq(adj) = 94,18%

Grouping Information Using Tukey Method and 95,0% Confidence

Oil Concentration	N	Mean	Grouping
0,50	6	2363,6	A
1,00	6	2224,2	A B
1,50	6	2165,6	A B
1,25	6	2131,5	B C
2,50	6	1948,5	C
3,00	6	1707,7	D

Grouping Information Using Tukey Method and 95,0% Confidence

Process	N	Mean	Grouping
HHP	18	2506,9	A
no HHP	18	1673,4	B

General Linear Model: Average T2 Value versus Oil Concentration; Process

Analysis of Variance for Average T2 Values, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Oil Concentration	5	1598724	1598724	319745	24,42	0,000
Process	1	6253025	6253025	6253025	477,56	0,000
Error	29	379715	379715	13094		
Total	35	8231465				

S = 114,427 R-Sq = 95,39% R-Sq(adj) = 94,43%

General Linear Model: Average T2 Value versus Oil Concentration; Process

Analysis of Variance for Average T2 Values, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Oil Concentration	5	1598724	1598724	319745	24,42	0,000
Process	1	6253025	6253025	6253025	477,56	0,000
Error	29	379715	379715	13094		
Total	35	8231465				

S = 114,427 R-Sq = 95,39% R-Sq(adj) = 94,43%

One-way ANOVA: Average T2 Values versus Oil Concentration

Source	DF	SS	MS	F	P
Oil Concentration	5	1598724	319745	1,45	0,237
Error	30	6632740	221091		
Total	35	8231465			

S = 470,2 R-Sq = 19,42% R-Sq(adj) = 5,99%

```
Grouping Information Using Tukey Method

Oil
Concentration  N    Mean  Grouping
0,50           6  2363,6  A
1,00           6  2224,2  A
1,50           6  2165,6  A
1,25           6  2131,5  A
2,50           6  1948,5  A
3,00           6  1707,7  A

Means that do not share a letter are significantly different.
Tukey 95% Simultaneous Confidence Intervals
```

One-way ANOVA: T2 Values versus Oil Concentration before HHP

```
Source             DF      SS      MS     F      P
Oil Concentration   5  646575  129315  5,22  0,009
Error              12  297438   24786
Total              17  944013

S = 157,4   R-Sq = 68,49%   R-Sq(adj) = 55,36%


Grouping Information Using Tukey Method

Oil
Concentration  N    Mean  Grouping
0,50           3  1920,9  A
1,50           3  1787,0  A B
1,00           3  1777,9  A B
1,25           3  1696,3  A B
2,50           3  1501,9  A B
3,00           3  1356,4    B
```

One-way ANOVA: T2 Values After HHP versus Oil Concentration

```
Source             DF       SS      MS      F      P
Oil Concentration   5  1003259  200652  77,26  0,000
Error              12    31165    2597
Total              17  1034424

S = 50,96   R-Sq = 96,99%   R-Sq(adj) = 95,73%
```

Grouping Information Using Tukey Method

Oil Concentration	N	Mean	Grouping
0,50	3	2806,20	A
1,00	3	2670,59	A B
1,25	3	2566,57	B
1,50	3	2544,14	B
2,50	3	2395,13	C
3,00	3	2059,01	D

Table B. 10. Correlations Between Chemical Analyses for Oil and Emulsion Samples

Correlations: DPPH; FRAP; PEROXIDE VALUE; Total Phenolic Content for Oil Samples

	DPPH	FRAP	PEROXIDE VALUE
FRAP	1.000		
	*		
PEROXIDE VALUE	-0.257	-0.257	
	0.304	0.304	
Total Phenolic C	0.349	0.349	-0.092
	0.156	0.156	0.717

Cell Contents: Pearson correlation
P-Value

Correlations: DPPH; FRAP; Peroxide Value; TBARS; TPC for Emulsions

	DPPH	FRAP	Peroxide Value	TBARS
FRAP	0.989			
	0.000			
Peroxide Value	-0.815	-0.824		
	0.000	0.000		
TBARS	-0.841	-0.875	0.813	
	0.000	0.000	0.000	
TPC	0.957	0.936	-0.668	-0.809
	0.000	0.000	0.002	0.000

Cell Contents: Pearson correlation
P-Value