INVESTIGATION OF STABILIZING PROPERTY OF CHITOSAN-*G*-CAFFEIC ACID COMPLEX IN OIL IN WATER EMULSION

by
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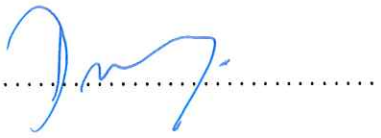
Assist. Prof. Dr. Derya Kahveci Karıncaoğlu ..... 

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To my parents…

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ABSTRACT

INVESTIGATION OF STABILIZING PROPERTY OF CHITOSAN-*G*-CAFFEIC ACID COMPLEX IN OIL IN WATER EMULSION

Emulsion is a mixture of two immiscible liquids, one liquid acts as disperse phase and another continuous phase, and oil in water (o/w) or water in oil (w/o) emulsions are most known emulsion types. Recently consumers have increasing demands for clean label, eco-friendly and plant derived (for vegetarians) products lead to seeking natural alternative solutions. In this thesis, the aim was that enhancement physiochemical properties of chitosan with caffeic acid modification to obtain eco-friendly emulsifier with antioxidant property and this study might give a perspective to literature about antioxidant and emulsion studies of modified chitosan; because, there is a gap about these studies in literature. For this purpose emulsification ability of chitosan, chitosan-*g*-caffeic acid and applicability and effectiveness of emulsification methods were evaluated. After the optimization studies, the dropwise method at 60 ℃ and normal Blk method that following 15, 000 rpm at 15 min was evaluated for 1, 1.5 and 2 percent (v/v) chitosan and 1 percent(v/v) chitosan-*g*-caffeic acid including emulsions. Also, 1 percent (v/v) chitosan and chitosan-g-caffeic acid based emulsions were evaluated against ionic strength and pH conditions. The emulsion characterization results showed that obtained emulsions mean droplet size were < 100 μm so emulsion type was conventional. Caffeic acid modification produced (IC_{50}) value as 2.5, 1.3, 0.6 mg/ml and (EC_{50}) value as 1.8, 0.3, 0.4 mg/ml for 5, 10, 20 mM caffeic acid containing chitosan-*g*-caffeic acid complexes respectively. The radical scavenging activity of caffeic acid modified samples (48.6 percent) reduced their activity in emulsion (4.9) but reducing power of samples (0.5 abs at 700 nm) increased in emulsions (1.9 abs at 700 nm). Caffeic acid modification caused to decrease in surface charge of emulsions from ~60 to ~20 mV. Also lowest viscosity values of chitosan modified samples were the reasons of higher creaming fraction values. These results were implied that both Ch and Ch-*g*-Ca had emulsifier and stabilizing activity and also Ch-*g*-Ca might be candidate for specific emulsion mixtures with its antioxidant property.

ÖZET

KİTOSAN-*G*-KAFEİK ASİT KOMPLEKSİNİN STABİLİZE ETKİSİNİN SU İÇERİSİNDE YAĞ EMÜLSİYONUNDA ARAŞTIRILMASI

Emülsiyonlar normal şartlarda birbirine karışamayan maddelerden oluşan yapılardır. Bu yapılarda bir sıvı devamlı fazı oluşturuken diğer sıvı dağılan fazı oluşturmaktadır. En çok bilinen emülsiyon çeşitleri arasında su içerisinde yağ emülsiyonu ve yağ içerisinde su emülsiyonu gösterilebilir. Son zamanlarda tüketicilerin çevreye dost, temiz etiketli ve doğal ürünlere olan ilgisi yeni emülgatör arayışına sebep olmuştur. Bu tez kapsamında chitosan maddesinin modifikasyonu ile hem çevre ile dost hem de antioxidant aktivitesi olan bir emülgatör üretilmesi planlanmıştır çünkü literatür çalışmalarına bakıldığında bu konu hakkında yeterli çalışma yapılmadığı gözlemlenmiştir. Bu amaçla kitosanın ve kafeik asit ile modifiye edilmiş kitosanın emülsiye edici etkisi ve farklı homojenizasyon metotlarının verimliliği ve uygulanabilirliği değerlendirilmiştir. Yapılan optimizasyon çalışmaları sonucunda 60 ℃ de ısı altında damlama methodu ve normal kitlesel karıştırma metotları ve ardından 15, 000 rpm hız ile 15 dakika yüksek hızlı karıştırıcı uygulamasına karar verilmiştir. Bu amaçla 1, 1.5 ve 2 yüzde (v/v) kitosan örneklerinden oluşan emülsiyonlar damla boyutu, krem faz oluşturma fraksiyonu ve hızı bakımından değerlendirilmiştir. Ayrıca 1 yüzde (v/v) kitosan ve kafeik asitle modifiye edilmiş kitosan örnekleri içeren emülsiyonlar iyonik güç ve pH koşullarına göre değerlendirilmiştir. Elde edilen damlacık boyut sonuçları (<100μm) elde edilen emülsiyonun klasik emülsiyon türünde olduğunu göstermiştir. Modifiye edilmiş kitosan örnekleri 5, 10, 20 mM kafeik konsantrasyonları için sırasıyla serbest radikal indirgeme aktivitesi için (IC_{50}) değerini 2.5, 1.3, 0.6 mg/ml olarak ve indirgeme gücü için (EC_{50}) değerini 1.8, 0.3, 0.4 mg/ml sahip olduğu bulundu. Serbets radikal aktivitesine sahip olan örnekler bu özelliği emülsiyon içinde zamanla kaybederek modifiye edilmiş kitosanın değeri olan yüzde 48.6 dan 4.9 değerine ulaşmıştır, öte yandan indirgeme gücü ise emülsiyon içinde zamanla artarak modifiye edilmiş madde miktarına göre 0.5 ten 1.9 absorbans değerine ulaşmıştır. Kafeik asit modifikasyonu yüzey yükünün ~60 mV tan ~20 mV değerine azalmasına, düşük viskosite değeri yüksek krem fazı oluşumuna sebep olmuştur. Sonuçlar Ch ve Ch-*g*-Ca nın hem emülsiye hem de stabilize edici etkiye sahip olduklarıını göstermiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

d_{10}	Number-weighted mean diameter
d_{32}	Surface-weighted mean diameter
d_{43}	Volume weighted mean diameter
r	Radius
ζ-Potential	Zeta potential
z-average	Mean droplet diameter
μ	Micrometer
λ	Wavelength
Abs	Absorbance
Blk	Bulk
Ch	Chitosan
Ch-g-Ca	Chitosan-*g*-Caffeic acid
DW	Dropwise
Minute	Min
$O_1/W/O_2$	Oil-in-water-in-oil emulsion
O/W	Oil-in-water emulsion
PSD	Particle Size Distribution
W/O	Water-in-oil emulsion
$W_1/O/W_2$	Water-in-oil-in water emulsion

1. INTRODUCTION

Emulsion is a mixture of two immiscible liquids, one liquid acts as disperse phase and another continuous phase, and oil in water (o/w) or water in oil (w/o) emulsions are most known emulsion types. In a well-designed emulsion system, dispersed phase presents as small droplets in continuous phase. Emulsions are colloidal systems that would be involved in production of various products such as mayonnaise, salad dressings, coffee creams in food industry, pharmaceutical products, and so on. However, any changes in environmental conditions such as pH, temperature, ion content might promote some physiochemical mechanisms such as creaming, fluctuation, and coalescence that will disrupt emulsion stability during processing or storage. Therefore, this lead to need for an emulsifier that will increase emulsion kinetic stability [1]. The important point is that choosing most effective emulsifier to form emulsion and protect stability against environmental changes [2]. An emulsifier is a surface-active material that has a hydrophilic part, soluble in water phase, and hydrophobic (lipophilic) part, soluble in oil phase. In this study, physiochemical features of chitosan were improved by modification with caffeic acid to prepare alternative emulsifiers to commercial ones. The characterization of emulsions was done via determining of droplet size, electrical charge (ζ-potential), microstructure and creaming stability.

2. LITERATURE REVIEW

Emulsions consist of association of two immiscible compounds as in the form of two phases which are continuous phase in which dispersed phase presents as droplets. There are various applications of emulsion in industry such as in food industry as end products such as coffee creamers and cream liqueurs or as ingredient as in yoghurt and gelled systems [3] and encapsulation of functional agents such as flavor, color, antimicrobials and so on [4]. In pharmaceutical industry, emulsions are used for encapsulation of hydrophilic or lipophilic active compounds with the aim of protection from environmental conditions such as light, temperature, oxygen, acidity and so on [5]. It is possible to make classification of emulsion according the arrangement dispersed/continuous phase [6], droplet size range of emulsion (nano, micro, macro emulsion) [5], type of the emulsion system whether conventional emulsion or multilayer emulsion or droplet size distribution; monodisperse or polydisperse. Emulsions are made up from two different phases first one is called dispersed phase, second is called continuous phase. If the oil is dispersed phase, this emulsion type named as oil in water (O/W) such as milk, salad dressing, mayonnaise, and sauces and if water is dispersed phase and oil is continuous emulsion is named water in oil (W/O) emulsion such as butter and margarines [7].

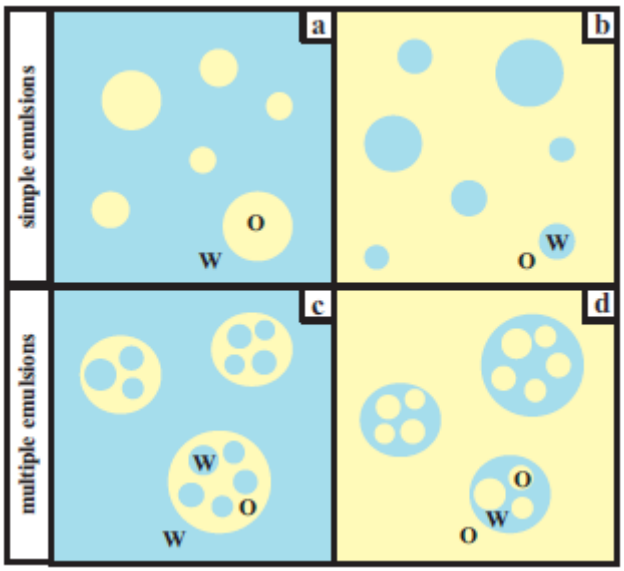

Figure 2.1. Illustration of a) oil-in-water, b) water-in-oil, c) water-in-oil-in -water d) oil -in-water-in oil emulsion [5]

Micro emulsions are thermodynamically stable systems, and stability of these systems caused mainly their low interfacial tension and low droplet size [5]. Generally, macro emulsions composed of oil, surfactant and water [4] and also they can include co-surfactants [5]. Having smaller droplet size than wavelength of light (λ=390-750 nm) causes the scattering of light thereby appearance of this type emulsion might be transparent or slightly turbid [4, 5]. Like micro emulsions, nano emulsions consisting of oil, surfactant, and water but this system is thermodynamically unstable compared to micro emulsions [4]. Also, they are transparent or slightly turbid because their droplet size is small (r< 100 nm) [4], (2-200 nm) [5]. Mini emulsions are in the 100-1000 nm droplet radii range [7]. The droplet size of macro emulsion can be between 0.1 and 100 μm and their colors are white due to ability of scattering light [5], 1000 nm-1000 μm [7]. Emulsions are thermodynamically unstable like nano emulsions but their appearance is turbid or opaque because their large droplet size (r > 100 nm) [4], 100 nm-100μm [6].

Besides conventional emulsions, multiple emulsions can be formed by using advanced processing techniques. Water-in-oil-in-water ($W_1/O/W_2$) and oil-in-water-in oil ($O_1/W/O_2$) are some examples of structurally complex emulsions [7]. This type of emulsions is useful for protection of labile ingredients and release of specific ingredients [8]

2.1. EMULSIFIERS

Emulsifiers are compounds that show their functions by dispersing emulsions uniformly and/or stabilizing them via increasing kinetic stability of emulsions [9]. Unlike stabilizers, emulsifiers are surface active compounds [9] thereby they are made up of lipophilic and hydrophilic parts to perform solubility both in non-aqueous (oil or fat) phase and aqueous phase [9, 10]. The emulsion formation and stabilization abilities of emulsifiers are attributed their interfacial and/or thickening characteristics [5]. The main characteristics of an emulsifier must be rapid reduction of interfacial tension during emulsification thus reduction of free energy [11], rapid adsorption to the interface and, protection the droplets against destabilization mechanisms [2]. Emulsifiers exhibit their functions by producing thin coating around the droplets and protecting them from aggregation via generating repulsive forces between droplets [7]. Low molecular weight surfactants, proteins,

phospholipids, polysaccharides, and derivatives of them are some examples of utilized emulsifiers in food industry [7].

2.2. NATURAL EMULSIFIERS

In industry, chemical surfactants such as Tween 80, Tween 20, and sodium dodecyl sulfate (SDS) have been used as emulsifier in various areas. Surfactants have surface active components consisting of hydrophobic and hydrophilic parts and protect emulsion stability with aid of electrostatic repulsion between charged droplet surfaces to overcome interfacial tension between two phases. However, recently consumers increasing demands for clean label, eco-friendly and plant derived (for vegetarians) products lead to seeking natural alternative solutions.

Proteins, phospholipids and polysaccharides are natural biopolymers and their emulsifying properties have been studying recently. Improvement in the emulsifier activity of natural polymer based compounds like in polysaccharides and proteins can be achieved by some modification of hydrophobic side groups or by the degree of hydrophobicity of the amino acid residues [9].

Whey and casein are protein based and lecithin is phospholipid based natural emulsifiers [12]. Proteins form electrically charged thin interfacial layers and both proteins & phospholipids protect emulsion stability with electrostatic repulsion which is more susceptible to environmental changes in pH, salt than steric repulsion. Additionally, in the case of polysaccharides surface activity characteristic can come from molecule origin or can be given by attachment of non-polar chemical group and, protein components [2]. With the change in pH of the medium, polysaccharides and proteins can be charged thereby adsorption of these compounds on the droplet interface can lead to electrostatic repulsion. Among the natural emulsifiers polysaccharides perform slow adsorption to droplet surfaces due to their higher molecular weight and produce thick hydrophilic interfacial layer [5, 9] and their utilization in different process conditions is more suitable compared to protein and small molecule emulsifiers because later ones can be a pH, ionic and temperature sensitive [13]. This large size of polysaccharides can be an advantage which will cause steric repulsion thereby stabilization of emulsion or can be a disadvantage that will lead depletion attraction and induce flocculation or aggregation [9] that accelerate

destabilization of emulsion system. Stabilization of emulsion by polysaccharide based emulsions causes formation negative charged droplets because of anionic groups such as sulfate and carboxyl on polymer chain [6]. Polysaccharides such as cellulose, starch, and chitin are other natural biopolymers that produce thick hydrophilic interfacial layers and behind electrostatic repulsion they show steric repulsion to protect stability of emulsions [1, 10]. Chitin is a linear polysaccharide, polymer of β-(1-4)-N-acetyl-D-glucosamine monomers and found in cell walls of fungi & algae, exoskeleton's of insects, mollusks and crustacean's shells. Chitosan is linear polysaccharide and N-deacetylated form of chitin, composed of β-(1-4) linked 2 amino-2 deoxy -D-glucose monomers. Also, it is only polycationic polymer in nature [14, 15]. Although, chitin is not soluble in organic solvents and water due to the strong intermolecular hydrogen bonds while chitosan can be soluble in aqueous acid solutions; because of free amino groups. Biodegradable, biocompatible and non-toxic properties of chitosan increase opportunity of usage in many fields such as in food industry as antioxidant, antimicrobial, thickener and stabilizing agent, edible films and dietary fibers, in pharmaceutical for drug delivery systems, in biomedical for artificial skin and bones [14] and in waste water treatments systems as coagulants [16]. Although there were capability of using chitosan as native for various aim, grafting of functional compounds on chitosan molecule enhances its properties and increases its functionality. Caffeic acid [17], chlorogenic acid [18], hydroxycinnamic acid [19], epigallocatechin-3-gallete (EGCG) [20, 21], sodium phosphorylate [22], tripolyphosphate [23] can be listed as functional compounds were used in chitosan modification studies. Recently, there have been several studies that focus on emulsifying or stabilizing feature of chitosan can be found in literature. These studies were based on chitosan itself or combination with other compounds. For example, Schulz, Rodriguez [15] showed chitosan could produce w/o/w double emulsion without any surfactant within economical perspectives. Del Blanco, Rodriguez [24] and Rodriguez, Albertengo [25] studied the impact of deacetylation degrees of chitosan on its emulsifying capacity by measuring of droplet size, viscosity, conductivity, and ageing behavior. Some researches were focused on chitosan stabilizing activity with presence of another emulsifier or surfactant; for instance, Laplante, Turgeon [26] determined emulsion stability of chitosan with different molecular weight and degrees of deacetylation in the presence of whey protein and also Speiciene, Guilmineau [27] studied effect of chitosan concentration on o/w emulsion stabilized with whey. Klinkesorn and Namatsila [28] presented that stabilizing ability of low molecular weight chitosan on

low acid tuna o/w emulsion in the presence of Tween 80 and Klinkesorn and McClements [29] studied effect of chitosan concentration and molecular weight on lecithin stabilized tuna o/w emulsion. Mun, Decker [30] showed effect of chitosan molecular weight and deacetylation degree on o/w emulsion in the presence of Tween 20 and SDS. On the other hand, recent researches were aimed to study emulsifier activity of modified chitosan for example the emulsifier efficiency of phosphorylated chitosan in o/w evaluated and in drug delivery systems [22] and Wei and Gao [18] modified chitosan with chlorogenic acid and tested its stabilizing property on β-carotene emulsion.

2.3 EMULSIFICATION

Emulsification process can be separated two classes as high energy techniques and low energy techniques [6]. High-speed mixer, colloid mill, high-pressure valve homogenizer, microfluidizers, and sonication are some examples of mostly preferred traditional emulsification devices [2, 6]. High energy homogenization method is based on high amount of new liquid interface [2] and intense disruptive forces [6]. However, during the emulsification process, it is possible to dissipation of energy as heat [2] and strong shear stress may give rise to coalescence of the dispersed phase. On the other hand, low energy homogenization method produces small droplets in the present of change in solution or environmental conditions and two examples of this method are phase inversion or spontaneous [6]. There are several factors that must be considered when choosing a homogenization technique such as site of the process it can be a laboratory or factory, physicochemical properties of start compounds and final products (i.e., sensitive compounds like protein), desired droplet size, working volume, economical aspects of process [11].

2.4. DROPLET CHARACTERISTICS

Droplet and its characteristics such as droplet size distribution, concentration, charge, interactions are main important factors that have effect on emulsion stability. Emulsification parameters such as pressure, time, temperature, number of passes, and instrument type, emulsifier features such as concentration, type, viscosity of continuous

phase, interfacial tension are factors that have effects on emulsion droplets [7]. The quantity of oil droplet in an emulsion system has critical effect on physicochemical, sensory, and nutritional characteristics of emulsion [7, 11]. The average droplet size and particle size distribution (PSD) are important parameters that give information about stability of emulsion. Although it is difficult that formation of monodisperse droplets which means that all droplets are the same size [11], but narrow droplet size distributions can be produced by adjusting homogenization conditions such as pressure, type of instrument, number of passing, oil to emulsifier ratio. Droplet size distribution of an emulsion system can be evaluated via using droplet concentration versus droplet size profile [11]. Unlike monodisperse emulsions, if the droplets of an emulsions are in different sizes it is called polydisperse emulsion and can be subdivided to three classes in terms of including peak number as monomodal (single peak), bimodal (double peak), and multimodal (multiple peak) [11]. Droplet charge impacts droplet interactions and it depends on emulsifier type and concentration and solution characteristics used in emulsion [7, 11]. While the anionic surfactants gain negative charges to droplets, cationic surfactants give positive charge [6]. Another significant emulsion structure is interfacial layer which is a combination of oil, water, emulsifiers and other components and it creates a boundary layer between the oil and water phase [7, 11]. Its properties such as thickness affect the stability, physicochemical and sensory features of an emulsion system [11].

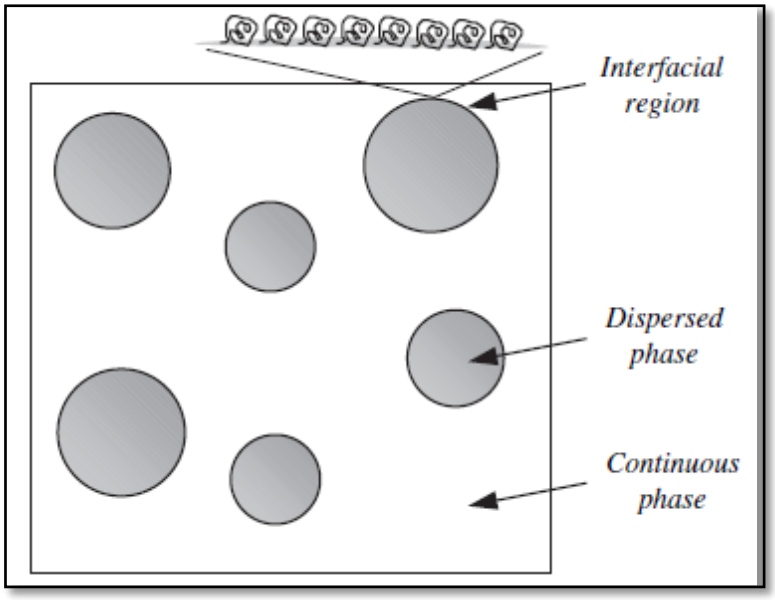

Figure 2.2. Schematic representation of main emulsion parts [31]

2.5. DROPLET INTERACTIONS

In an emulsion system there are several droplets interactions, van der Waals, electrostatic, steric, and hydrophobic interactions, hydrogen bonding, depletion which have impact on stability and physiochemical properties of emulsions [7, 11]. The sign, magnitude and range of these droplet interactions are important parameters that affect overall interaction between droplets [11]. The droplets interaction might be easily influenced by droplet characteristics, continuous characteristics and interfacial properties [7]. According the type and magnitude of these interactions droplets have tendency either to merge (coalesce), associate or present as individual. The van der Waals interaction is generally attractive and long range [11]. Electrostatic interaction is caused by charged droplets of emulsions that coated by ionic surfactants and it is long range and repulsive [11]. Increasing in magnitude of surface potential leads to increasing in strength of electrostatic repulsion but decreasing is observed as the concentration or valence ions of continuous phase increases due to screen effect of counter-ions on charges between droplets [11]. Steric repulsion is short range and is occurred during overlap of interfacial layers of two approaching droplets [10]. Macromolecules that are adsorbed at the interface and are soluble in continuous phase cause the steric repulsion [9]. Insufficient adsorption of droplets by emulsifiers can give rise to an attractive hydrophobicity interaction between droplets of emulsion and this type interaction might be strong and long range [11].

Table 2.1. Summary of most important droplet interactions [11]

Type	Sign	Magnitude	Range	Major Factors Influencing
Van der Waals	A	S	L	Refractive index, dielectric constant, ionic strength
Electrostatic	R or A	W-S	S-L	Droplet charge, pH, ionic composition
Steric	R	S	S	Solvent quality, interfacial thickness, interfacial packaging
Depletion	A	W-M	M	Excluded species, size, concentration
Hydrophobic	A	S	L	Surface hydrophobicity, temperature

Sign : Attractive (A), Repulsive (R); Magnitude: Weak(W), Medium (M), Strong (S); Range: Short (S), Medium (M), Long (L)

2.6. DESTABILIZATION MECHANISMS

There might be several factors that will be deterministic for stability of emulsions such as emulsifier nature, concentration, pH, ionic strength, or emulsification process conditions such as temperature, pressure, process duration. Throughout the emulsification process interface between dispersed phase and continuous phase is established by the mechanical energy supplied by an emulsification machine [9] and this lead to raise in interfacial free energy [5]. Emulsifier in an emulsion system is used to decrease interfacial tension between continuous and dispersed phases [9]. However, emulsion systems have tendency to decrease this interfacial contact are and free energy by phase separation [5]. Therefore, during storage or shelf life of emulsions experience some physiochemical mechanisms, flocculation, coalescence, gravitational phase separation (creaming or sedimentation), Ostwald ripening, that will lead to instability of emulsions.

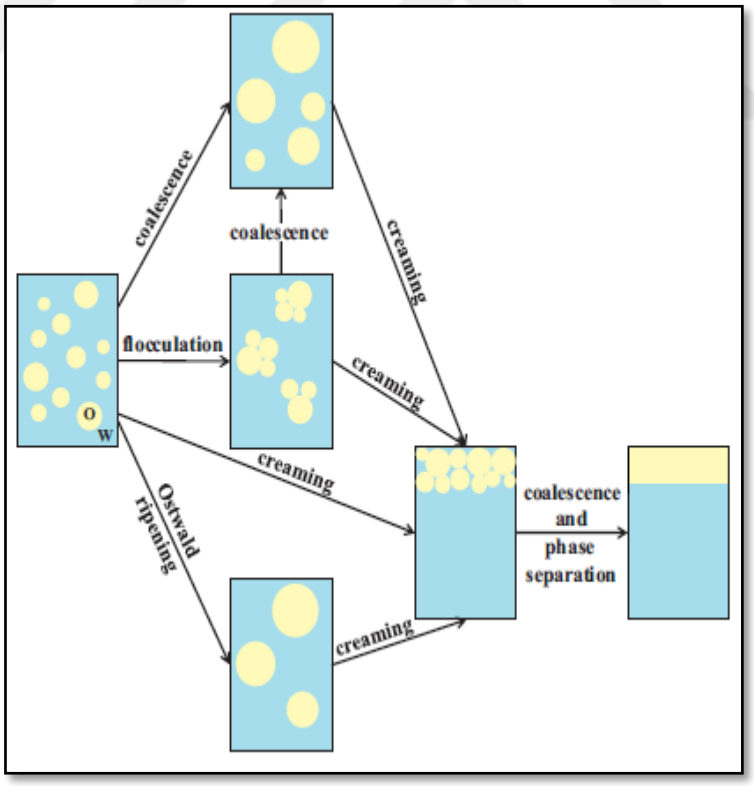

Figure 2.3. Destabilization mechanisms of oil-in-water emulsion

Flocculation and coalescence are most important destabilization mechanisms observed in emulsion systems [5].

2.6.1. Flocculation

Simple definition flocculation is that aggregation of dispersed phase droplets [9] and main driving force is attractive force between droplets [5]. Depletion and bridging mechanisms can be way of flocculation in emulsion stabilized by biopolymers [5]. Depletion flocculation can occur in the presence of excess biopolymer or non- adsorbed small surfactants or organic or inorganic nanoparticles [5]. Macromolecules which do not have affinity for the interface and dissolved neutral polysaccharides can be a reason for depletion attraction in an emulsion [9]. Bridging flocculation is caused adsorption of one biopolymer molecule by at the more than emulsion droplets. Flocculation can induce sedimentation in water in oil (W/O) emulsion or creaming in oil in water (O/W) emulsion, and coalescence [5, 9]. Although, some flocs can be damaged by shaking easily, reversible, some flocs cannot be disrupted, irreversible, thereby causes formation of coalescence in emulsion droplets [5].

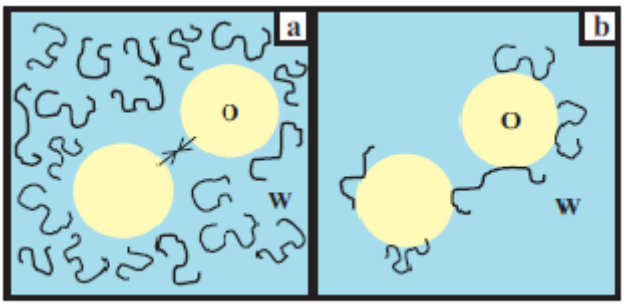

Figure 2.4. Representation of a) depletion and b) bridging flocculation in the presence of polymer [5]

2.6.2. Coalescence

Coalescence is another important destabilization mechanism, destabilization of emulsion begins by decreasing the oil-water interface and free energy. As the coalescence occurs small dispersed phase droplets merge and large droplets form due to less free energy and finally phase separation can be observed. Creaming induces coalescence process thereby utilization of appropriate emulsifiers that prevent drop-drop contact by steric or electrostatic repulsion and increasing viscosity of continuous phase might be some solution to prevent coalescence [9].

2.6.3. Creaming/Sedimentation

After emulsification droplets of emulsion can move upward or downward through the dispersion due to density differences of continuous and dispersed phase [5]. If the droplets density is lower than continuous phase, droplets move upwards and creaming occurs. However, if the droplets density is higher than external phase, droplets migrate downwards and sedimentation occurs. Creaming or sedimentation rate can be decreased via increasing continuous phase viscosity, decreasing the density difference between two phases and decreasing dispersed phase droplet size [9]. Additionally, as the concentration of droplet increases, creaming rate decreases [7].

2.6.4. Ostwald Ripening

Ostwald ripening is diffusion of dispersed phase through continuous phase because of solubility of dispersed phase in continuous phase, and droplet contact is not observed during this mechanism [5]. As a result, oiling off and phase separation are possible conclusions that related with these destabilization mechanisms [5].

Among these mechanisms flocculation, creaming and sedimentation are reversible mechanisms of movement of droplets because it is possible to change situation by applying external force. But Ostwald ripening and coalescence are non- reversible mechanisms that cause droplet size rise [5].

2.7. AIM OF THE STUDY

Emulsions are colloidal systems that consisting of mixture of two immiscible liquids which one is dispersed, and another is continuous phase. Oil in water (o/w) and water in oil (w/o) are most known examples of these systems. The colloidal systems are used in various areas for different purposes. In industry, chemical based emulsifiers such as Tween 80, Tween 20, SDS or animal origin proteins whey, casein or phospholipid based lecithin are mostly used as emulsifiers. However there is an increasing demand of consumers for all-natural, label and environmentally friendly ingredients and substances and this lead to replacement of chemical emulsifiers with natural ones. Although, protein based emulsifiers

such as whey and casein are natural but their higher price, being animal origin, and limited processing conditions restricted their usage. On the other hand, polysaccharides are most abundant natural biopolymers and derived mostly from plants (starch, cellulose) or from waste products (chitin). Also, unlike chemical surfactants and other natural biopolymers, polysaccharides show both steric and electrostatic (electro steric) repulsion for emulsion stability. Chitosan is a deacetylated form of chitin, composed of β-(1-4) linked 2 amino-2 deoxy -D-glucose monomers, and is only cationic polysaccharide in nature. Chitosan is non-toxic, biodegradable, and biocompatible biopolymer and it is most abundant polymer in nature after cellulose. Amino groups on its polysaccharide backbone give it advantage of chemical modifications. With grafting it is possible to introducing functional compounds into chitosan backbone to enhance its solubility and antioxidant features. In this study, it was aimed to enhance physiochemical properties of Ch with caffeic acid modification to obtain eco-friendly emulsifier with antioxidant property and emulsion preparation without any synthetic polymers just using chitosan and its derivative as emulsifier. Additionally, three distinct emulsification methods were evaluated to investigate influence of order of mixing on adsorption ability of chitosan and chitosan-g-caffeic acid derivative. For this purpose formed emulsions were characterized by dynamic light scattering for size and surface charge of emulsion droplets, by optical microscope for microstructure of droplets and by visual observation for creaming layer. The output of this study might give a view about relation the antioxidant activity and emulsifier or stabilizing activity of modified chitosan and obtained results might increase opportunity of using modified chitosan as single emulsifier or stabilizing agents in emulsion systems.

3. MATERIALS AND METHODS

3.1. MATERIALS

Chitosan (from shrimp shells, practical grade, 417963), Caffeic acid (purum, ≥ 95 percent HPLC, 60020) EDAC (N-(3-Dimethyaminopropyl)-N'-ethylcarbodiimide hydrochloride, E7750) Ethanol (absolute, ≥99.8 percent GC), Sodium hydroxide (assay 98-100.5 percent, 06203), Hydrochloric acid (assay 36.5-38 percent, 07102), Acetic acid (Fluka, 45731, Assay>99.8 percent, Germany), DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), Potassium phosphate monobasic, Potassium hexocyanoferrate, Trichloroacetic acid (TCA), Iron (III) chloride were purchased from Sigma-Aldrich Company and Extra Virgin olive oil was purchased from local market.

3.2. METHOD

3.2.1. Optimization of Chitosan Concentration & Emulsification Method

In this part of the study, optimization studies were conducted with different emulsification methods such as sonication, homogenization, thermal treatment, ultrasonic water bath, dropwise addition, and their combinations. Also chitosan concentration effect was examined . Optimization results were evaluated based on mean droplet size of emulsions. Initially, ultrasonication (Bandelin Sonoplus, HD 2200) was applied as 4 cycle x 52 percent power to o/w emulsions stabilized with 3, 2, 1.5, 0.5, and 0.1 percent (v/v) Ch solutions at 1 and 5 minute (min). After the ultrasonication process, homogenization with high shear blender (Ultra Turrax, T18, Germany) at 25, 000 rpm speed for 5 min and its combinations with 1 and 5 min ultrasonication treatments were performed. Thermal treatment was applied as heating emulsion mixtures to 65 and 80 °C in water bath (WiseBath) and additionally 5 min ultrasonication (4 cycle x 52 percent) and homogenization with high shear blender at 25, 000 rpm speed for 5 min were performed, respectively.

For chitosan dropping and homogenization combination method, Ch was added drop by drop with pasteur pipette on rest of the mixture to obtain o/w emulsion including 1.5 percent (v/v) Ch. After dropping process homogenization was applied at 15, 000 rpm for 9, 12 min separately. Unless otherwise specified for all droplet size measurements were done after dilution at 1:100 rate in 1 percent (v/v) acetic acid.

Last optimization method was decided as heating and combination of heating and homogenization methods. Studies were carried out on 1 percent (v/v) Ch concentration including emulsion mixture and homogenization at 15, 000 rpm speed for 30 min, heating at 60 °C for 90 min in ultrasonic water bath (Bandelin, Sonorex) and then homogenization at 15, 000 rpm speed for 15 min, and heating at 60 °C for 90 min and homogenization for 30 min were performed respectively on individual prepared samples. Before droplet size measurements emulsion samples were diluted at 1:100, 1:500, 1:5000 rates in 1 percent (v/v) acetic acid solution.

3.2.2. Optimization of Chitosan Concentration

The effect of Ch concentration and emulsification method on the stability and physicochemical characteristics of extra virgin olive oil in water emulsion (o/w) were tested by checking creaming stability, droplet size, droplet charge and visual observation by an optical microscope.

3.2.2.1. Chitosan Solution Preparation

Ch solutions were prepared in 1 percent (v/v) acetic acid solutions at 2 and 3 percent (w/v). The solutions were stirred overnight at room temperature to ensure complete dissolving. Then, impurities were removed by centrifugation at 4, 000 rpm and 4 °C for 30 min.

3.2.2.2. Emulsification

Emulsification was achieved by two-step processes that were mixing and homogenization. After mixing of emulsifier and oil in water, one of the homogenization methods that were

described in below was performed. 1 percent (v/v) acetic acid was used as water phase and extra virgin olive oil was used as oil phase for all experiments.

3.2.2.2.1. Dropping of Chitosan in oil

Firstly, extra virgin olive oil and acetic acid at 1 percent (v/v) final concentration was blended and Ch solution was added dropwise by using a graduated brute. Then mixture was stirred under appropriate rpm speed and heated at 60 ℃ to obtained homogen mixture.

3.2.2.2.2. Dropping of oil in Chitosan

Ch solution and acetic acid at 1 percent (v/v) final concentration was blended and extra virgin olive oil was added dropwise by using a graduated brute. Then mixture was stirred under appropriate rpm speed and heated at 60 ℃ to obtained homogen mixture.

3.2.2.2.3. Bulk Mixing of Chitosan and oil

Ch solution, extra virgin olive oil and acetic acid at 1 percent (v/v) final concentration were all blended together and stirred under appropriate rpm speed and heated at 60 ℃ to obtained homogen mixture.

For all emulsions, 1, 1.5, and 2 percent (v/v) Ch concentrations were evaluated and continuous phase and dispersed phase ratio was adjusted to 9:1 in 50 ml volume. After mixing step, emulsions were homogenized immediately by high-speed hand homogenizer (Ultra Turrax, T18, Germany) at 15, 000 rpm speed for 15 min. All emulsion processes were performed as three replicates for each Ch concentration.

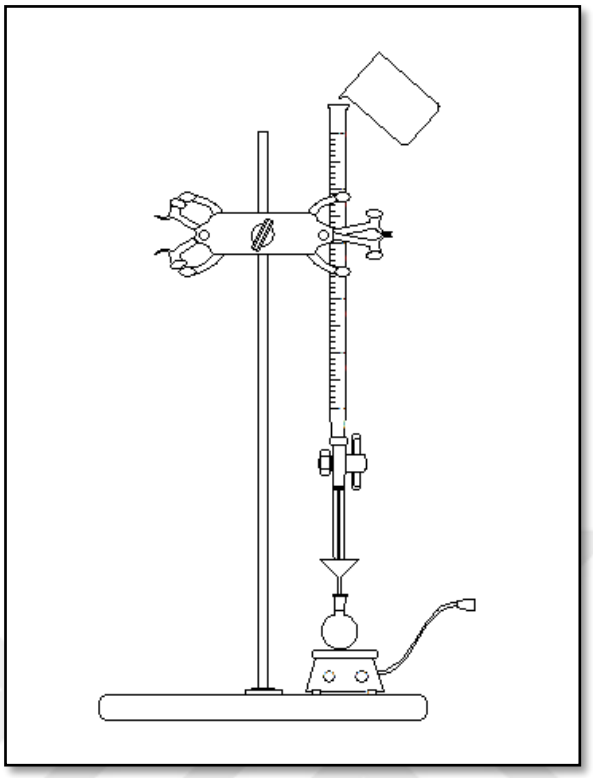

Figure 3.1. Representation of dropping process

3.2.3. Droplet Size Determination

After homogenization, the droplet diameter of emulsions was measured by dynamic light scattering instrument (Zetasizer, Nano-Zetasizer, Malvern) immediately. Before the measurement, emulsion samples were diluted at 1:100 rate in 1 percent (v/v) acetic acid to avoid multiple effects of light scattering. Each measurement was done as three replicates and results were evaluated by instrument software and expressed as mean droplet diameter (*z*-average). The results were analyzed as mean value ± standard deviation [32].

3.2.4. ζ- Potential Determination

ζ- potential measurement of oil droplets were done with particle electrophoresis instrument (Zetasizer, Nano-Zetasizer, Malvern). Before the measurement samples were diluted at 1:100 rate in 1 percent (v/v) acetic acid to avoid multiple effects of light scattering. Each

measurement was done as triplicate. The results were analyzed as mean value ± standard deviation [33].

3.2.5. Creaming Stability

After emulsification process, samples were stored in glass bottles at room temperature and creaming layer formation was observed visually during storage time. Creaming layer height was measured at specific time intervals and expressed as a percentage of total emulsion sample height and creaming rate. The results were analyzed as mean value ± standard deviation [34].

3.2.6. Microscope Observation

The droplet microstructures of the emulsions were assessed by an optical microscope (Axiolab, Carl, Zeiss). For microscopic observation samples were diluted at 1:10 in 1 percent (v/v) acetic acid solution. Microscopic images of a selected area were observed at 10, 20, 40 X objective magnifications.

3.2.7. Preparation of Chitosan-*g*-Caffeic Acid Modification

Chitosan-*g*-Caffeic acid complex was formed to increase antioxidant activity of complex by grafting caffeic acid to chitosan by using EDAC coupling reagent as connector of amino group in chitosan to carboxyl acid group of caffeic acid (17). Ch-g-Ca complexes were prepared as different grafting ratios (5, 10, 20 mM).

Ch (50 mM) was dissolved in 1percent (v/v) acetic acid under stirrer overnight to ensure complete dissolving. Centrifugation was performed to eliminate impurities at 4, 000 rpm speed for 30 min. Caffeic acid (5,10,20 mM) and EDAC (50 mM) were added to Ch solution, respectively, and pH of the mixture was adjusted to 5.0 with 1 M NaOH. The mixture was incubated at room temperature for 3 hours under stirrer to complete reaction. The reaction mixture was placed in dialysis bags (10 kDa MWCO) and they were put in distilled water at 4 °C for dialysis process. Dialysis process was repeated three times by

changing water with 12-18 hours intervals. After dialysis, centrifugation at 14,000 rpm speed for 30 min was done and additional filtration process was applied to obtain a pure sample. Then the sample was stored at -80 °C overnight and lyophilization was performed and product was protected at 4 ℃ until use [17].

3.2.8. Measurement of Modification Degree of Chitosan-*g*-Caffeic Acid

The spectrophotometric measurement was used for determination of bound caffeic acid. A 10 mg/ml concentration of sample was prepared in 1 percent (v/v) HCl. A 250 µl of sample was added on HCl (1 percent (v/v); 250 µl). Concentrated HCl (2 percent (v/v); 4.5 ml) was added to this mixture. Absorption measurement was done at 320 nm by using HCl (1 percent (v/v)) as blank after 15 min incubation time. Spectrophotometric measurements were assessed by using a caffeic acid standard curve that made by using different caffeic acid concentration range between 0.00225 to 0.36 mg/ml. Caffeic acid binding ratio was calculated with Equation 3.1.

$$\text{Binding Ratio (\%)} = (\text{Caffeic acid in sample (mg)} / \text{sample (mg)}) \times 100 \qquad (3.1)$$

3.2.9. Measurement Antioxidant Activity of Chitosan-*g*- Caffeic Acid

3.2.9.1. Radical Scavenging Activity Measurement

Chitosan-*g*-caffeic acid complexes with 5, 10, 20 mM caffeic acid concentrations were dissolved in 1 percent (v/v) HCl as 10 mg/ml concentration. For each caffeic acid concentration samples were diluted at different ranges, 3-1, 2.5-0.25, 1-0.1 mg/ml for 5, 10, 20 mM respectively. Onto samples (200µl) 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) solution (0.2 mM in ethanol; 600 µl) was added and stirred immediately. After 30 min incubation time at darkness and room temperature samples were transferred to 96 well plate and absorbance measurement was done at 540 nm by using 1 percent (v/v) HCl as blank. The DPPH- scavenging activity was evaluated according the Equation 3.2.

$$\text{Activity (\%)} = [1-(\text{Absorbance of Sample} / \text{Absorbance of Blank})] \times 100 \qquad (3.2)$$

Half-inhibition concentration (IC_{50}) was evaluated as the concentration of sample at which 50 percent of free radical of DPPH reduced [17].

3.2.9.2. Reducing Power Measurement

Chitosan-*g*-caffeic acid complexes with 5, 10, 20 mM caffeic acid concentrations were dissolved in 1 percent (v/v) HCl as 10 mg/ml concentration. For each caffeic acid concentration samples were diluted at different ranges, 3-1, 2.5-0.25, 1-0.1 mg/ml for 5, 10, 20 mM respectively. Samples were mixed with sodium phosphate buffer (0.2 M; pH 6.6 ;1 ml). After the addition of potassium hexocyanoferrate (1 percent (w/v); 1ml) reaction started and continued in water bath at 50 ℃ for 20 min. Reaction was completed with the addition of trichloroacetic acid (TCA) (10 percent (w/v); 1 ml) . Centrifugation was done at 7,000 rpm for 20 min and supernatant was diluted (1 ml) with distilled water (1ml) and iron (III) chloride (0.1 percent (w/v); 0.2 ml) was added on diluted supernatant. After 5 min incubation time at room temperature, sample transferred 96 well plate and absorbance measurement was done at 700 nm by using 1 percent (v/v) HCl as blank. The high absorbance value is indication of high reducing power activity. The effective concentration (EC_{50}) was evaluated as the concentration required to obtain a 50 percent antioxidant activity [17].

3.2.10. Chitosan-*g*- Caffeic Acid Emulsion Preparation

For this emulsion formation 20 mM caffeic acid including chitosan modification solution was prepared as 2 percent (w/v) in 1 percent(v/v) HCl. The solution was stirred overnight at room temperature to ensure complete dissolving. Then above mentioned dropping chitosan in oil, dropping oil in chitosan and bulk mixing chitosan and oil emulsification methods were performed. 1 percent (v/v) acetic acid was used as water phase and extra virgin olive oil was used as oil phase for all experiments. Additionally to see effect of ionic strength and pH prepared emulsion samples were treated with 100 and 200 mM NaCl and pH of the emulsion were adjusted to pH 5 and 9 via 1 M NaOH and 1 M HCl. All emulsion processes were done as two replicate. The emulsions were evaluated by checking creaming stability, droplet size, droplet charge and visual observation by an optical microscope depending on storage time. Optical microscope observation was achieved at 5 X objective

magnification. Droplet size and droplet charge measurements were done as three replicates and the results were analyzed as mean value ± standard deviation.

Also for comparison, the emulsion was formed by using 0.5 (w/v) % tween 80 (35) with bulk mixing method without heat treatment at 10, 000 rpm speed homogenization. The emulsion oil concentration was 10 (v/v) % and water was used as continuous phase.

3.2.11. Measurement Antioxidant Activity of Chitosan-*g*- Caffeic Acid Emulsion

The radical scavenging activity and reducing power activity of oil in water emulsion samples including chitosan-*g*-caffeic acid were measured according 3.2.9.1. and 3.2.9.2. protocols to see effect of caffeic acid effect against storage time. The difference from above protocols was that the samples were diluted at 1:10 in 1 percent (v/v) acetic acid for radical scavenging activity and used as is for reducing power measurements. For radical scavenging activity 1 percent (v/v) HCl was used as blank. Measurements were performed as two replicates and the results were analyzed as mean value ± standard deviation.

3.2.12. Measurement Viscosity of Emulsions

The viscosity measurements of emulsion containing 1 percent (v/v) chitosan and 1 percent (v/v) chitosan-*g*-caffeic acid samples were done one day later from formation with Brookfield DV3T Rheometer by using zero number spindle at 4-5 rpm speed at ambient temperature [36].

4.RESULTS

4.1. OPTIMIZATION OF EMULSIFICATION

4.1.1. Ultrasonication

Ultrasonication caused reduction of the mean particle diameter of emulsion below 10 µm (Figure 4.1). While, the results of 1 min application varied between 8.6±1.3 and 1.7±0.3 µm, the results of 5 min process were between 6.9±1.2 and 4.8±1.0 µm. For 1 min application, with decreasing of Ch concentrations there is gradually decline in droplet size of emulsions as 8.6±1.3 to 1.7±0.3 µm. However, it was not the same for all chitosan concentrations of 5 min ultrasonication application. There was a directly proportional relation between 3, 2 and 1.5 percent (v/v) Ch concentration and droplet size of emulsions, 6.9±1.2, 6.1±1.0 and 4.8±1.0 µm. But for 0.5 and 0.1 percent (v/v) Ch concentrations droplet size values increased and there were 6.2 ±0.5 and 5.6 ±0.5, respectively. In addition to this, there is no process time influence on both 2 and 1.5 percent (v/v) Ch emulsions. The results of 2 percent (v/v) Ch emulsion were 6.2 ± 0.2 and 6.1 ± 1.0 µm and the results of 1.5 percent (v/v) Ch emulsion were 4.7 ± 0.1 and 4.8± 1.0 µm for one and 5 min ultrasonication, respectively. As a result, the most efficient result is observed in 0.1 percent (v/v) Ch emulsion with 1 min ultrasonication, 1.7±0.3 µm.

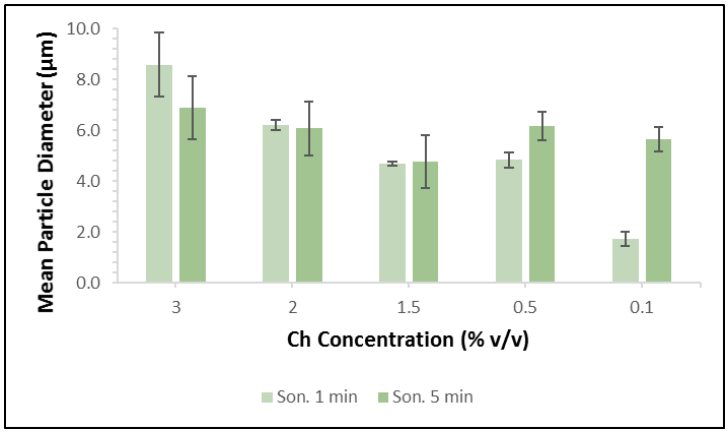

Figure 4.1. Effect of ultrasonication on droplet size of emulsions containing different Ch concentrations

4.1.2. Ultrasonication & Homogenization

The ultrasonication and homogenization combination treatments exhibited that when the homogenization applied alone, droplet sizes ranged between 7.3 ± 2.5 and 3.8 ±0.3 µm, for homogenization and 1 min ultrasonication between 7.2±0.6 and 5.7±1.1 µm and for homogenization and 5 min ultrasonication between 7.0±0.9 and 3.3±0.4 µm (Figure 4.2). When the results of homogenization without additional process compared with previous ultrasonication minimum droplet size results, it was seen that homogenization cause the decline in droplet size from 6.9 ±1.2 to 5.3 ±0.9 µm for 3 percent (v/v) Ch emulsion and from 6.1 ± 1.0 to 3.8± 0.3 µm for 2 percent (v/v) Ch emulsion. For 0.5 percent (v/v) Ch concentration result was nearly the same, it was changed from 4.8±0.3 to 4.9±1.1 µm, but it could not be said for other Ch concentrations. One min ultrasonication with homogenization increased mean droplet size of for all concentrations except 1.5 percent (v/v) Ch. However, 5 min ultrasonication with homogenization result in reduction droplet size of emulsion except, 0.1 percent (v/v) Ch. Although, there was no direct correlation between process methods and Ch concentrations but in general it was revealed that performing 5 min ultrasonication after homogenization was the most efficient method to reduce droplet size of emulsion.

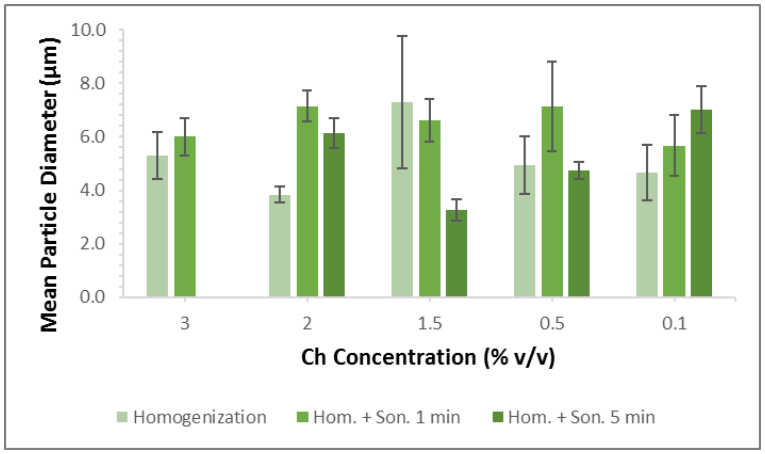

Figure 4.2. Effect of homogenization and ultrasonication combinations on droplet size of emulsions containing different Ch concentrations

4.1.3. Ultrasonication and Homogenization with Thermal Treatment

The thermal treatment results of 65 and 80 °C were varied between 6.3±0.4 and 4.5±0.7 µm and 8.5±2.2 and 4.7±1.3 µm, respectively. Although there might be effect of additional methods, ultrasonication and homogenization, results showed lower temperature heat treatment is more efficient to reduce droplet size of emulsions. Also, for 65 ℃ heat treatment, there was a gradually decreasing trend in droplet size with decreasing percent (v/v) Ch concentrations. When concentrations of Ch decreased from 3 to 0.5 percent (v/v), droplet size reduced from 6.3±0.4 to 4.5±0.7 µm. Additionally, when results of previous 5 min ultrasonication without additional treatment, 6.9±1.2 and 4.8±1.0 µm, compared with additional 65°C heat treatment, 6.3±0.4 and 4.5±0.7 µm, it was seen that there was effect of heating. But for 80°C any regular trend was not examined. Also, adding of heat treatment up to 80°C was compared with homogenization process and results showed that heating up to 80°C increase droplet size of some emulsions such as for 2 percent (v/v) Ch emulsion from 3.8±0.3 to 8.5±2.2, µm and for 0.5 percent (v/v) Ch emulsion from 4.9±1.1 to 8.3±2.1 µm. For 1.5 percent (v/v) Ch emulsion results were nearly same, 7.3±2.5 and 7.4 ±0.6 µm.

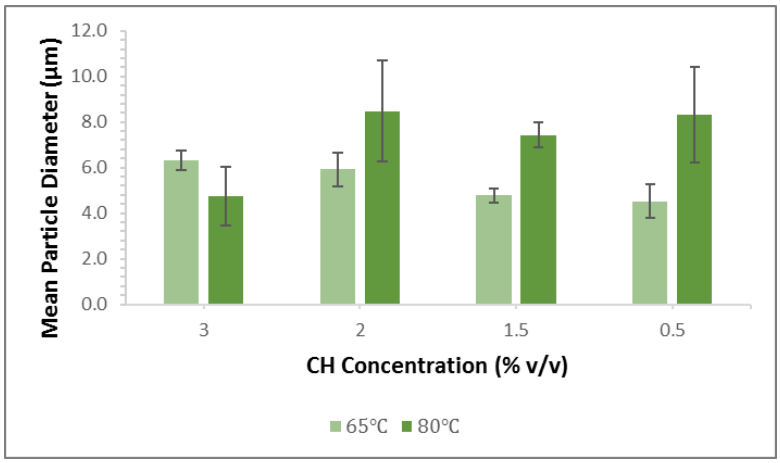

Figure 4.3. Effect of thermal treatment on droplet size of emulsions including different Ch concentrations

4.1.4. Homogenization & Thermal Treatment

The results of the chitosan dropping method showed that mean particle diameter of Ch dropping and 9 min. homogenization applied o/w emulsion was 10.4 ± 1.03 µm and Ch dropping and 12 min homogenization applied was 5.9 ±1.13 µm. According the results, there might be a converse relationship between homogenization time and mean particle diameter of oil droplets. Until this part of the optimization studies, various techniques and their combinations were applied. Among these methods thermal treatment at 65°C and then homogenization at 25, 000 rpm for 5 min led to better reduction of droplet size of emulsions than other methods as mentioned early section. Therefore, the optimization studies were followed by thermally treating and homogenization with high shear blender. Unlike previous heating parameters, heating at 60 °C in ultrasonic water bath was selected to get comparable results with literature and homogenization speed was reduced to 15, 000 rpm to increase availability of homogenization machine As seen in Figure 4.4, for both three methods droplet size results for 1:100 dilution rate did not exceed 10 µm like previous results. The result was 8.0±1.1 µm without heat treatment and mean particle size of emulsion reduced to 5.1±1.2 µm and 5.8±2.0 µm for 15 min and 30 min homogenization processes with heat treatment, respectively. Outcomes of this study represented that lower homogenization time with combination heating was enough to get satisfactory results. On the other hand, as seen in Figure 4.4, rising dilution rate could not give consistent results.

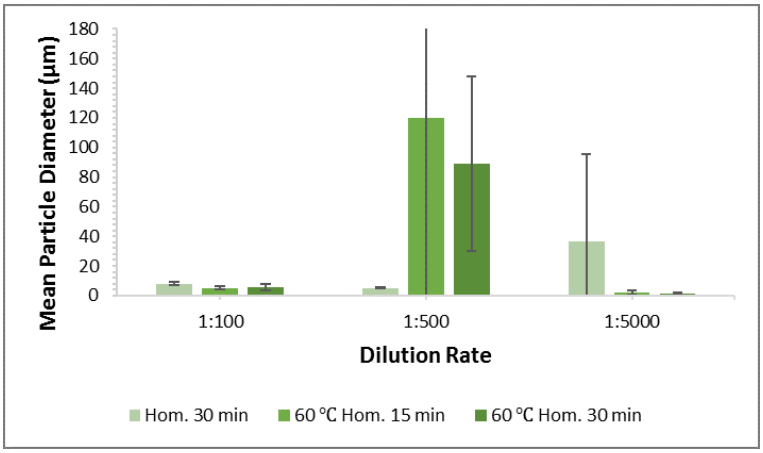

Figure 4.4. Effect of thermal treatment and homogenization combinations on droplet size of 1 percent (v/v) Ch emulsions

4.1.5. Chitosan Concentration Optimization

After preliminary studies for homogenization technique chitosan dropwise, oil dropwise and bulk methods following high-speed hand homogenization at 15, 000 rpm for 15 min were chosen as emulsification methods. The aim of trying dropwise method for chitosan and oil and bulk method is to discover most suitable method for adsorption of chitosan on oil droplet surfaces. These homogenization methods were examined for 1, 1.5 and 2 percent (v/v) chitosan concentrations. These methods were assessed in terms of droplet mean diameter, creaming fraction and rate, and also optical observation. Mean droplet diameter results of emulsions including 1, 1.5 and 2 percent (v/v) chitosan concentrations presented that Ch-DW method results gradually increased , 8.2, 8.3, 11.6 µm, with the rise in chitosan concentration (Figure 4.5). Similar to Ch-DW method, results of the O-DW method exhibited a rising trend as 8.1, 9.6, 10.8 µm as the chitosan concentration increased. Unlike the Ch-DW and Oil-DW methods, there was no a relation between mean droplet size results of Blk method and Ch concentration. For this technique, the smallest value was measured as 8.9 µm for 1.5 percent (v/v) Ch concentration, 9.4 and 10.9 µm were measured for 2 and 1 percent Ch (v/v) concentrations, respectively.

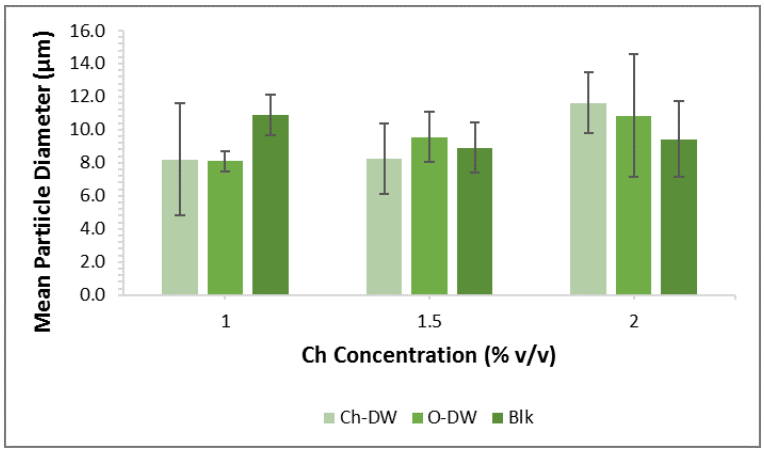

Figure 4.5. Effect of 1, 1.5 and 2 percent (v/v) Ch concentrations on droplet size of emulsions

Apart from emulsification method aspect, data can be evaluated in terms of Ch concentration for each method. For 1 percent (v/v) Ch concentration, Ch- DW and O-DW showed near results 8.2 and 8.1 µm respectively and Blk emulsification method lead to

higher mean droplet size, 10.9 µm. These results can be associated with creaming rate and creaming fraction values so emulsions were observed visually (Figure 4.9, 4.10, 4.11). Creaming fraction values for Ch-DW method ranged between 7.8 and 18.7 percent (Figure 4.6), for O-DW method were between 7.7 and 17.7 percent, and for Blk method were ranged between 9.6 and 19.2 percent. There might be a correlation between mean droplet size and creaming fraction values for 1 percent (v/v) Ch including emulsion for each method. Although there were some variabilities as seen in the Figure 4.6 at the intermediate storage days, when the initial and final creaming fraction values were compared similar behavior in mean droplet size were observed. O-DW method exhibited lowest creaming fraction values than other methods, Ch-DW method followed it and lastly higher creaming values were obtained by Blk method. Although for each method creaming fraction values increased day by day, creaming layer formation rate decreased as seen in the Figure 4.6. As the storage time increased the creaming rate values of three emulsification method approached each other and fixed at 1.3 for Ch-DW and O-DW methods and 1.4 for Blk method.

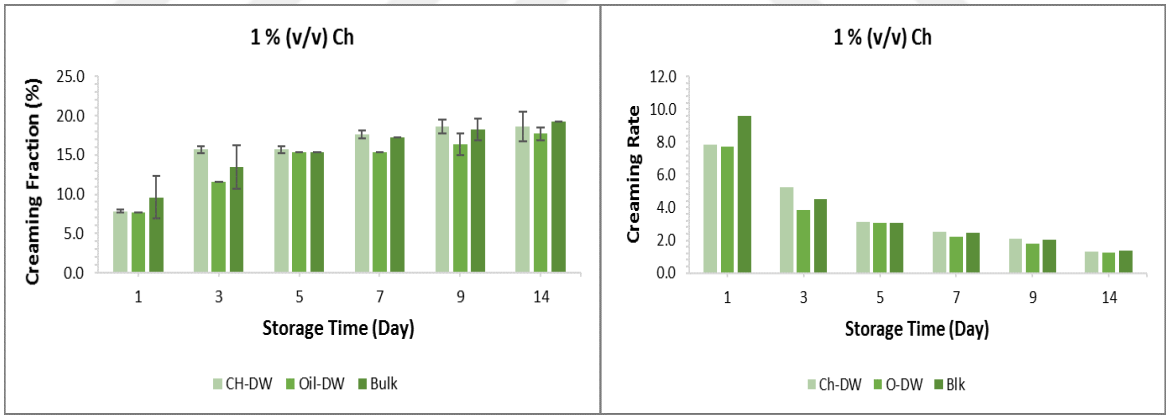

Figure 4.6. Creaming fraction (left) and creaming rate (right) of 1 percent (v/v) Ch including emulsions

Besides 1 percent (v/v) Ch concentration, the mean droplet size values of 1.5 percent (v/v) Ch including emulsion were compared and 8.3 µm mean droplet value obtained by Ch-DW method as smallest value. The Blk method followed with 8.9 µm and O-DW method followed with 9.6 µm as highest value. However the relation could not be observed between mean droplet size and creaming fraction values of 1.5 percent (v/v) Ch including

emulsions for all methods. In addition, although Ch-DW method produced smallest mean droplet diameter, creaming fraction values were higher than other two methods and ranged between 14 and 23 percent as seen Figure 4.7. While the creaming fraction values of Blk and O-DW methods showed some similarities during whole storage period, according the initial and last storage values it can be said that O-DW method created slightly highest creaming fraction values (10, 22 percent) than Blk method (9, 20 percent). Unlike Ch-DW method, the correlation between creaming fraction and droplet size was obtained for O-DW and Blk methods. As in the creaming fraction, creaming rate of Ch-DW method was higher than other methods which started with 4.8 and at the end of storage time fixed 1.2. O-DW and Blk methods showed some distinct values at the intermediate durations but initial and final values are the same, 3.2 and 1.1 respectively. As it appears, for all three methods although there was a rise in creaming fraction it decelerated as the storage time increased.

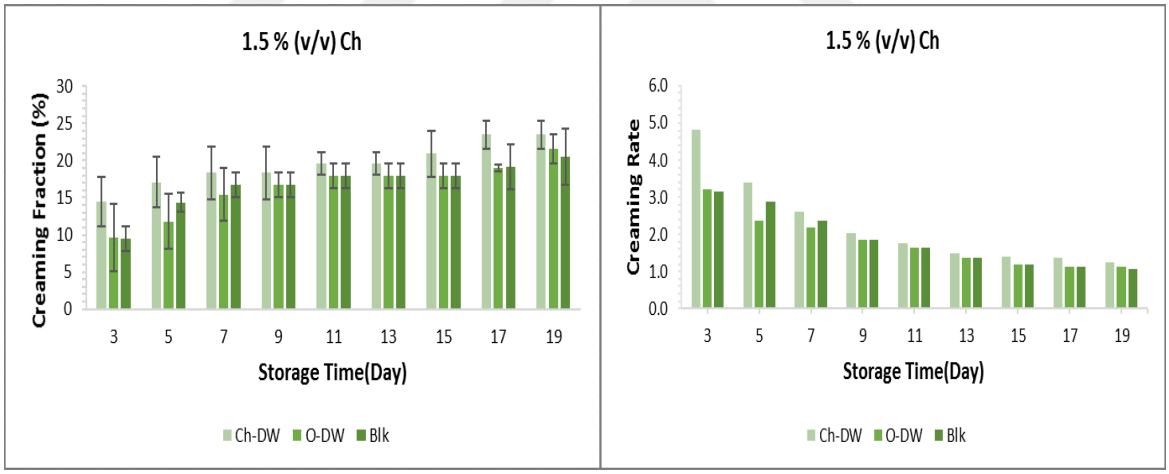

Figure 4.7. Creaming fraction (left) and creaming rate (right) of 1.5 percent (v/v) Ch including emulsions

The mean droplet size values of 2 percent (v/v) Ch including chitosan were 11.6, 10.8 and 9.4 μm for Ch-DW, O-DW, and Blk methods respectively. Creaming layer observation could be done at the 13. day for Ch-DW method but 21. Day for O-DW and Blk methods (Figure 4.8). As in the higher droplet size and earlier creaming layer formation, the highest creaming fraction was produced by Ch-DW method which was between 18-24 during storage time. The creaming fraction results of O-DW and Blk methods were 13-17 and 15

respectively. The 2 percent Ch including emulsions were achieved similar trend as in the droplet size, Ch-DW, O-DW and Blk methods produced highest to smallest values for two parameter. The creaming rate of Ch-DW method was ranged from 1.4 to 0.9 and there were some rises and falls between values of other two methods during storage time both of them fixed at 0.6.

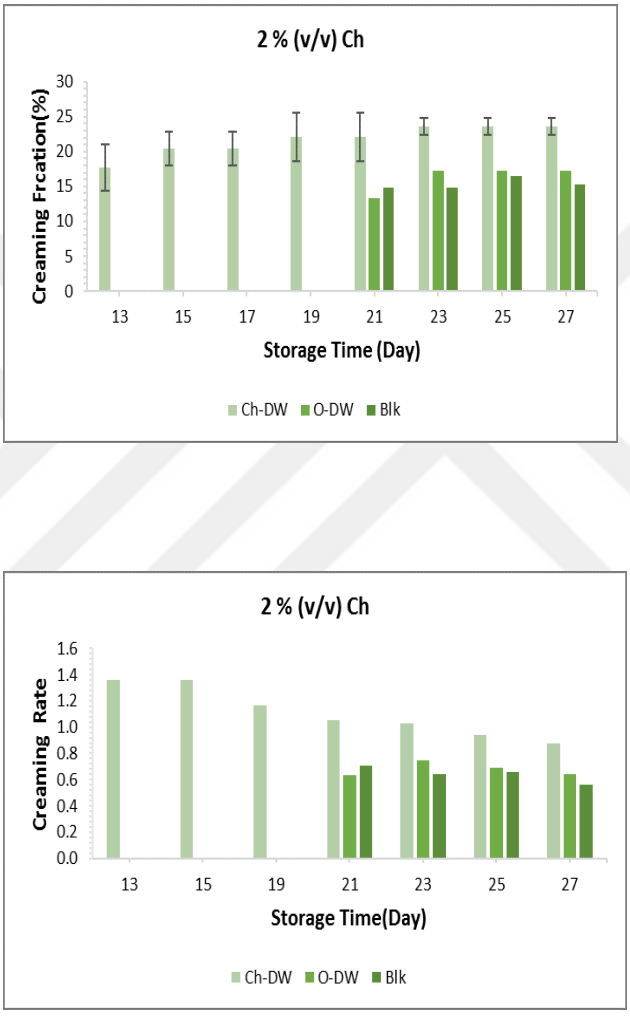

Figure 4.8. Creaming fraction (up) and creaming rate (down) of 2 percent (v/v) Ch including emulsions

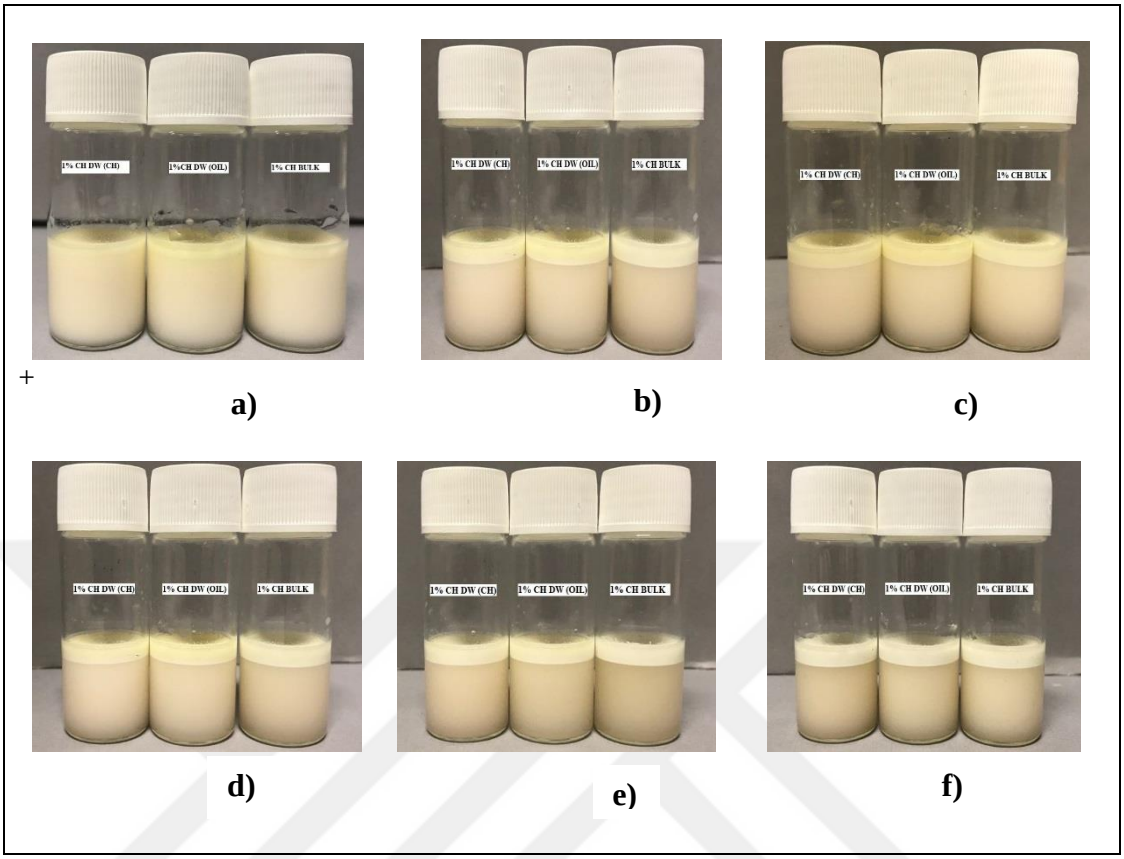

Figure 4.9. Visual appearance of 1percent (v/v) Ch including Ch-DW, O-DW, and Blk emulsions at day a) 1, b) 3, c) 5, d) 7, e) 9, and f) 14

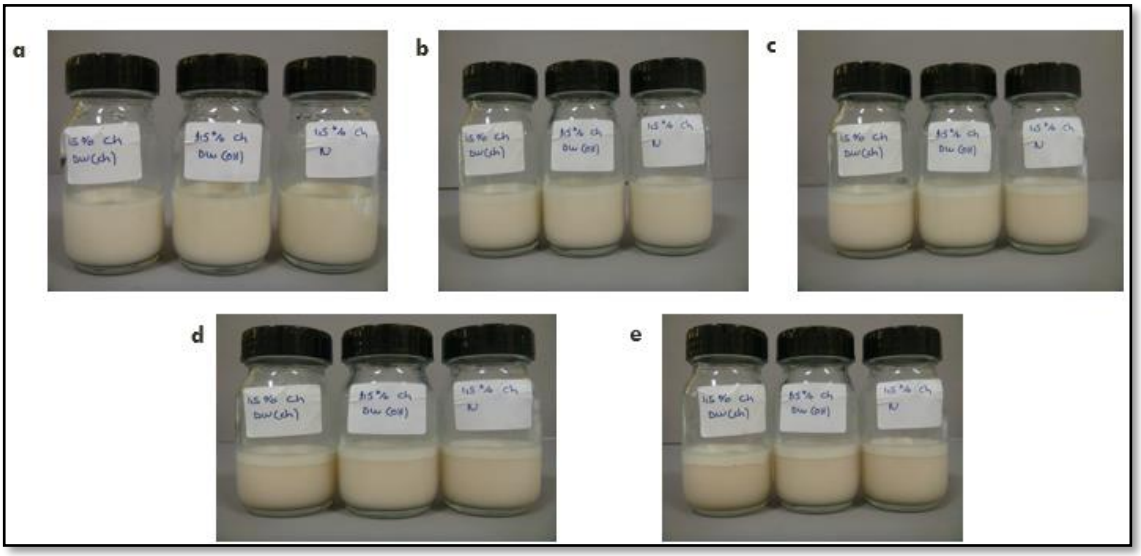

Figure 4.10. Visual appearance of 1.5 percent (v/v) Ch including Ch-DW, O-DW, and Blk emulsions at day a) 3, b) 7, c) 11, d) 17, and e) 21

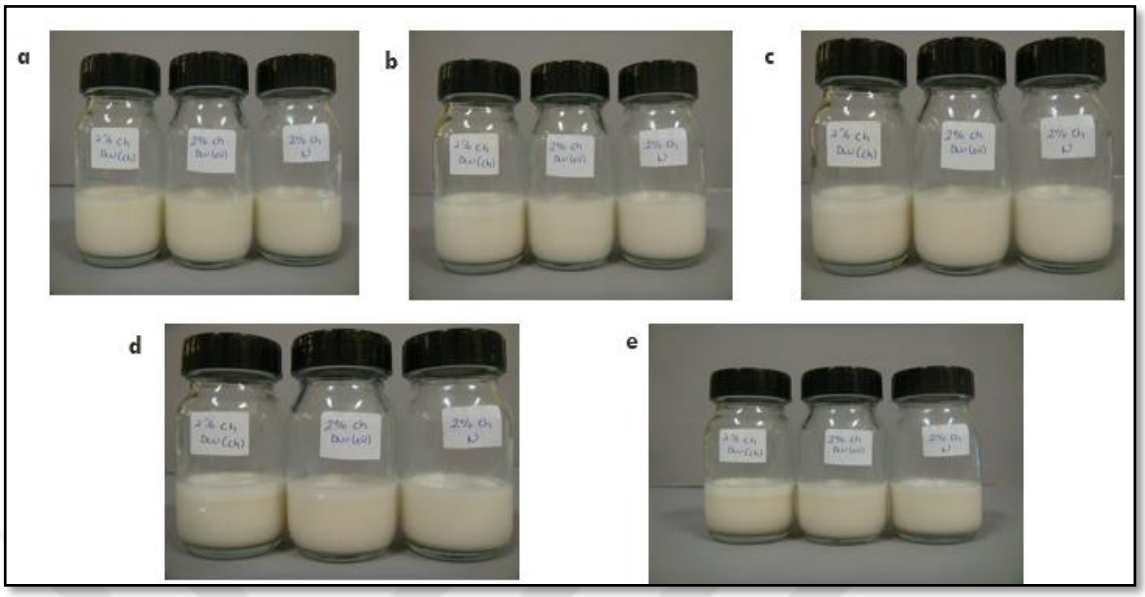

Figure 4.11. Visual appearance of 2 percent (v/v) Ch including Ch-DW, O-DW, and Blk emulsions at day a) 7, b) 13, c) 17, d) 23, and e) 27

The observation of chitosan concentration effect was continued with optical observation of emulsion samples with microscope. Each concentration was observed according the each emulsification method. Micrographs of emulsions presented that 1 percent chitosan concentration produced small droplets for all three methods (Figure 4.12, 4.13, 4.14) and it was possible to see in somewhere some bridging between droplets but there was not certain distinction between micrographs of emulsification methods. Similar to 1 percent chitosan, 1.5 percent chitosan produced small droplets as seen Figure 4.15, 4.16, 4.17, but the of bridging of droplets in Ch-DW method lead to formation of flocculation appearance in some places (Figure 4.15). Furthermore, O-DW and Blk mixing methods produced small droplets with bridging. Lastly, 2 percent chitosan concentration with Ch-DW method could not produce regular shape droplets (Figure 4.18), droplets merged together but O-DW (Figure 4.19) and Blk mixing (Figure 4.20) methods produced more regular droplets than Ch-DW methods but with the presence of bridging.

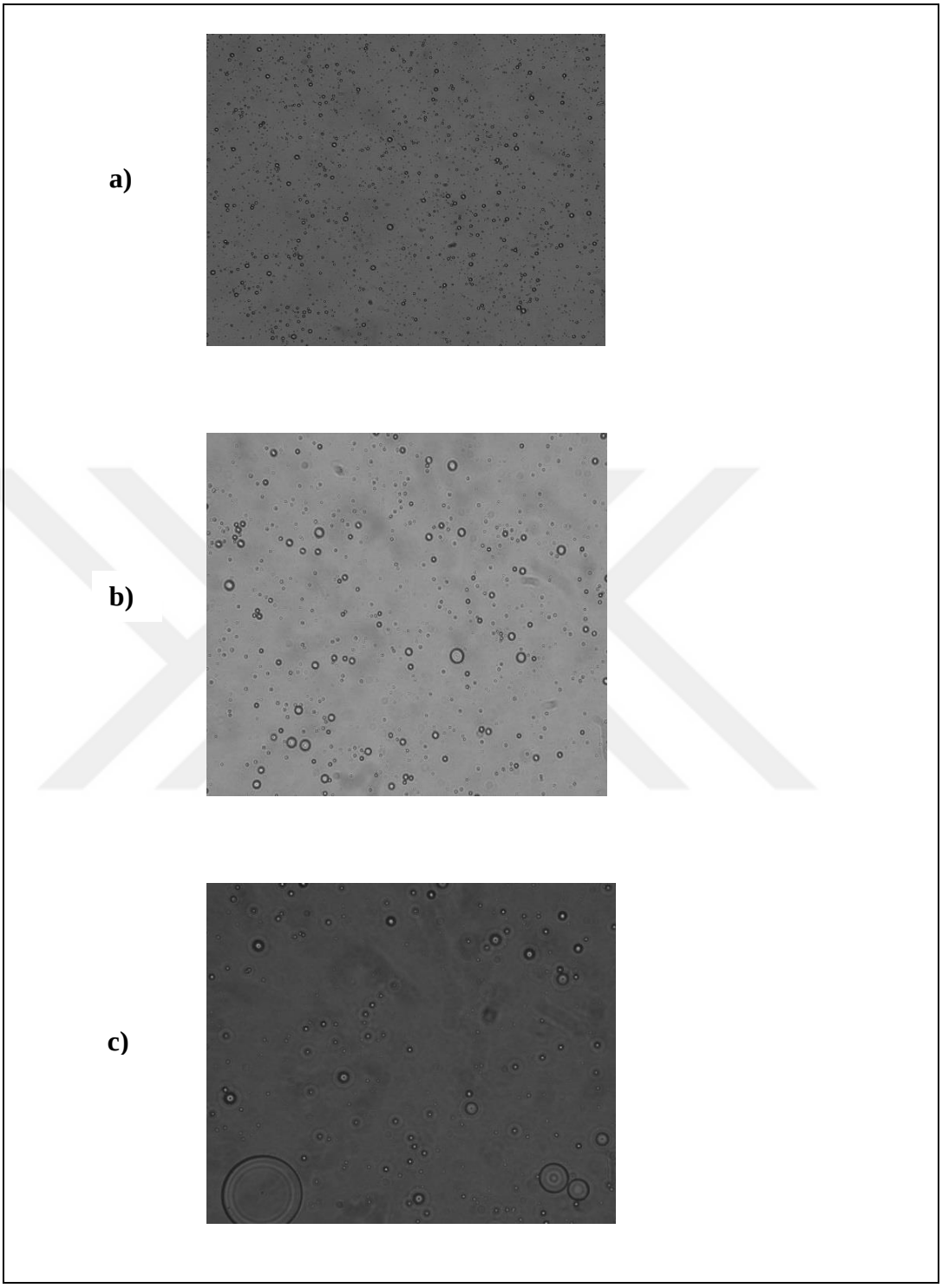

Figure 4.12. Optical micrographs of 1 percent (v/v) Ch including Ch-DW emulsions at a) 10, b) 20, and c) 40X objective magnifications

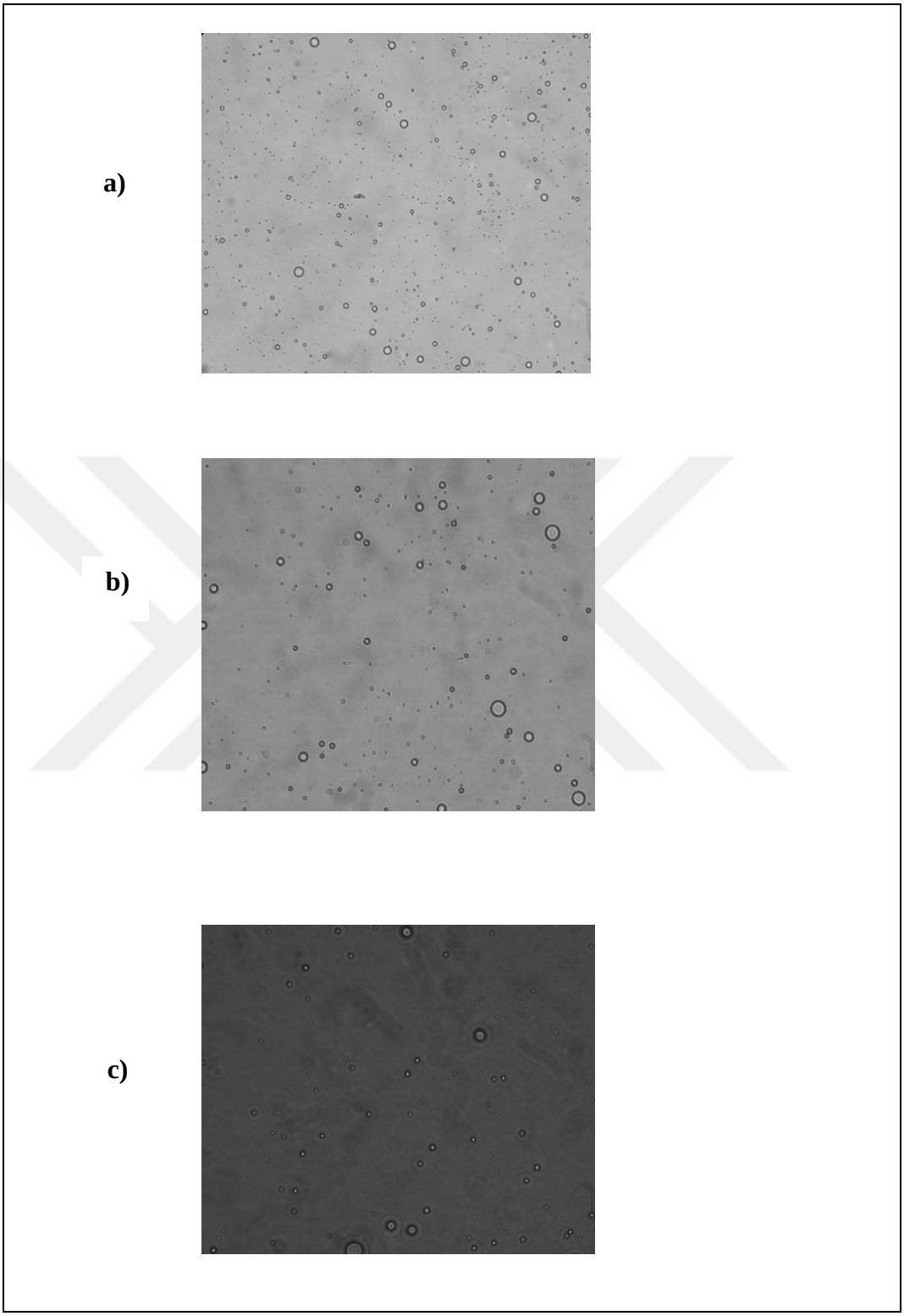

Figure 4.13. Optical micrographs of 1 percent (v/v) Ch including O-DW emulsions at a) 10, b) 20, and c) 40X objective magnifications

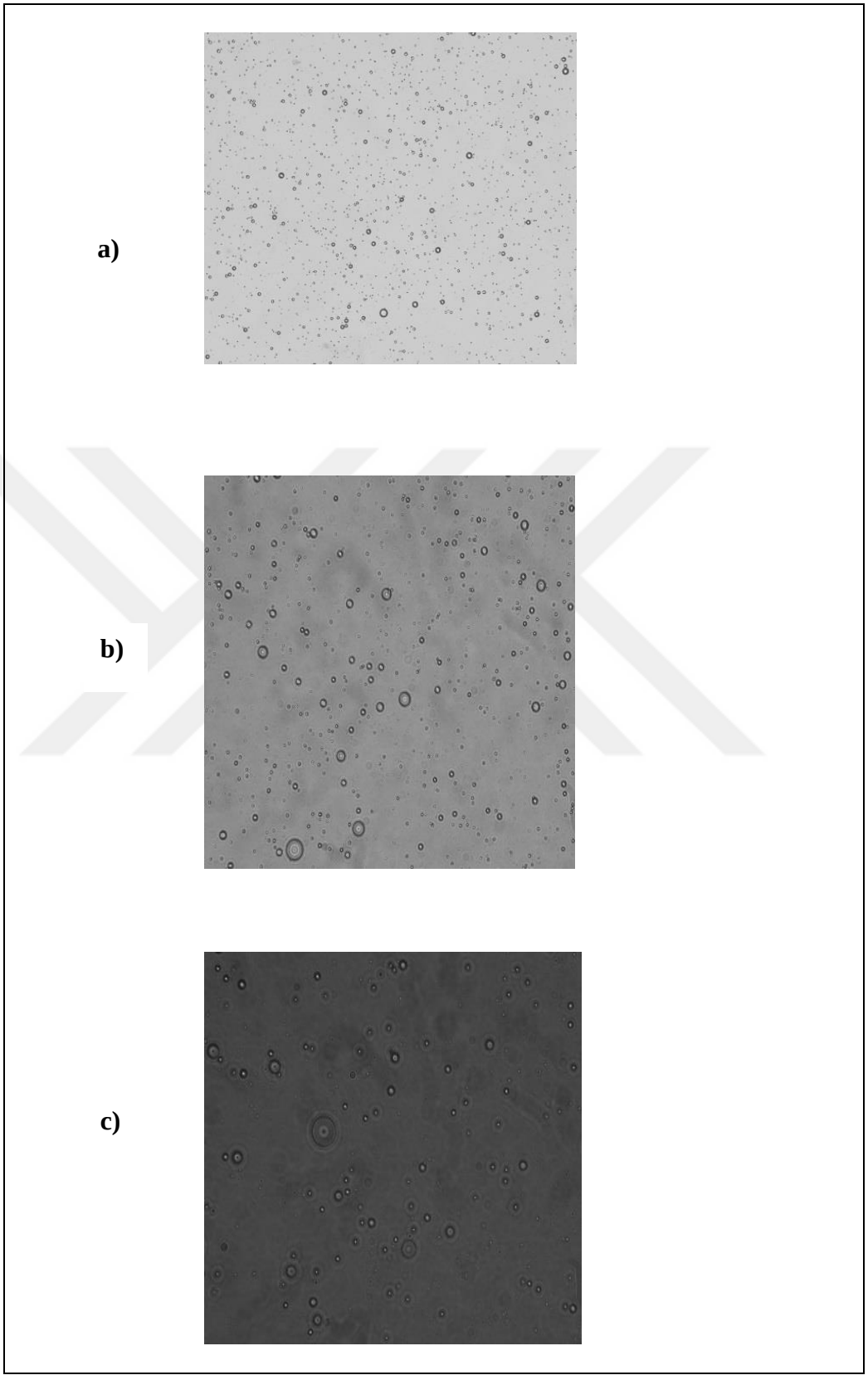

Figure 4.14. Optical micrographs of 1percent (v/v) Ch including Blk emulsions at a) 10, b) 20, and c) 40X objective magnifications

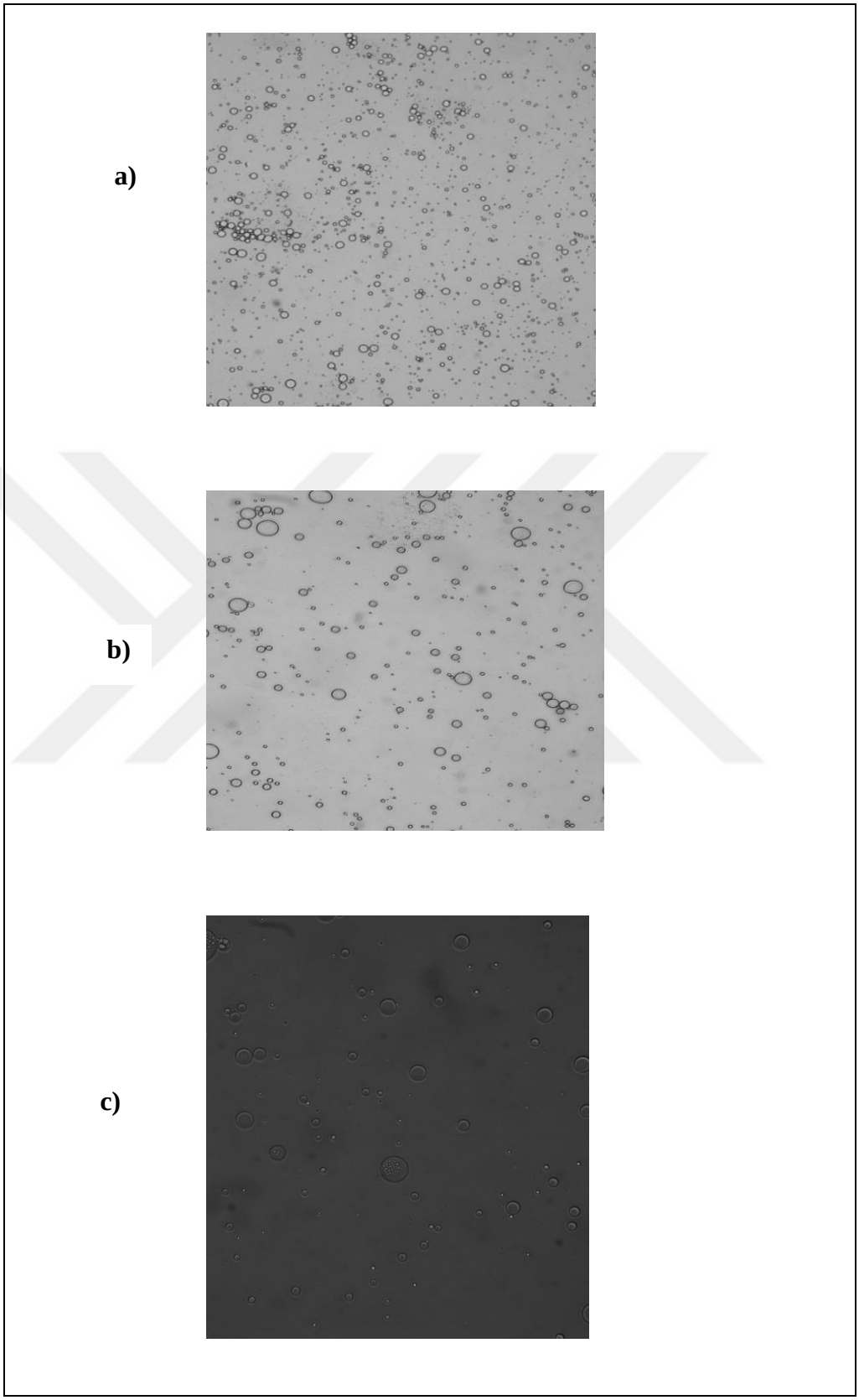

Figure 4.15. Optical micrographs of 1.5 percent (v/v) Ch including Ch-DW emulsions at a) 10, b) 20, and c) 40X objective magnifications

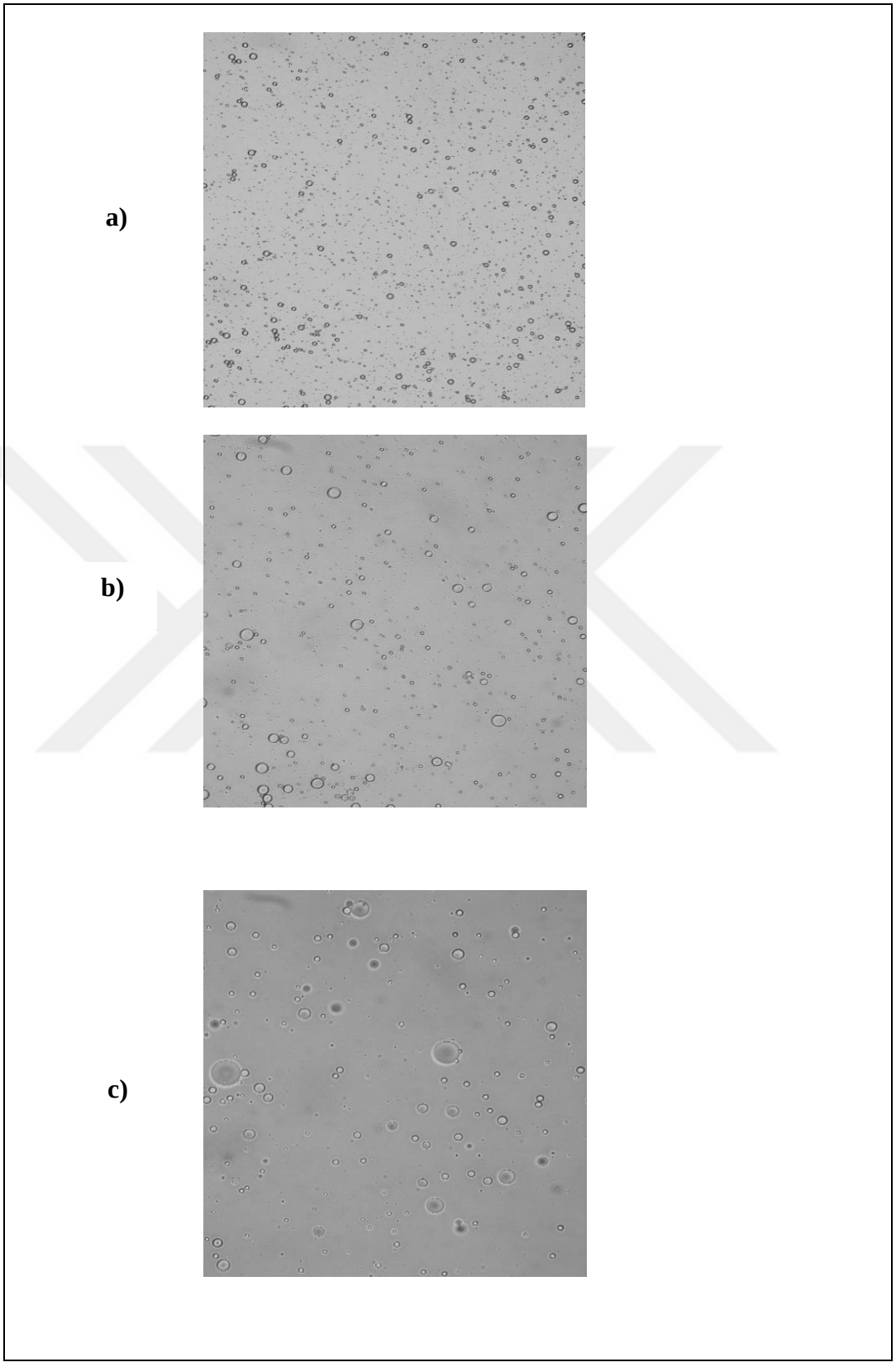

Figure 4.16. Optical micrographs of 1.5 percent (v/v) Ch including O-DW emulsions at a) 10, b) 20, and c) 40X objective magnifications

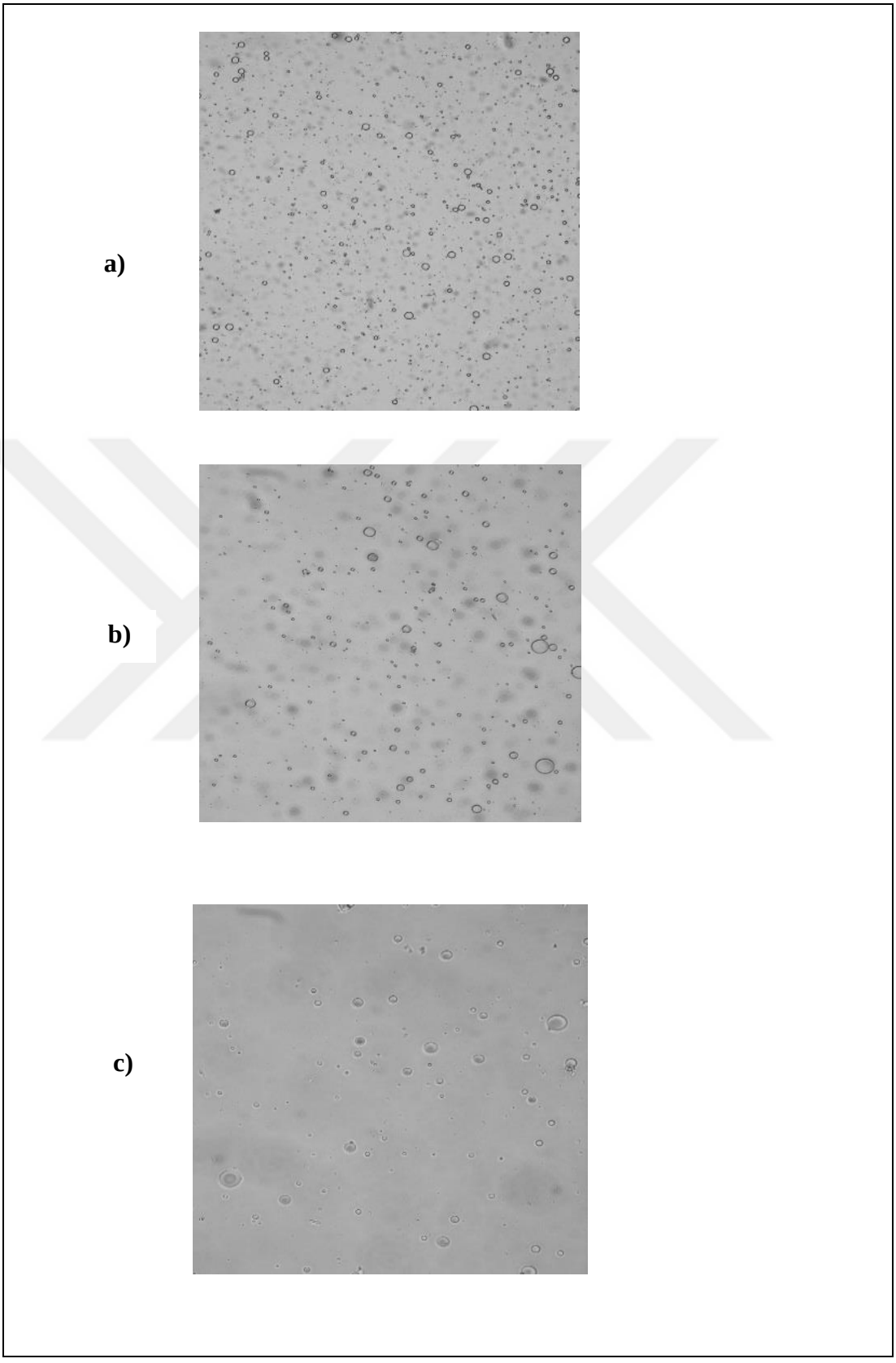

Figure 4.17. Optical micrographs of 1.5 percent (v/v) Ch including Blk emulsions at a) 10, b) 20, and c) 40X objective magnifications

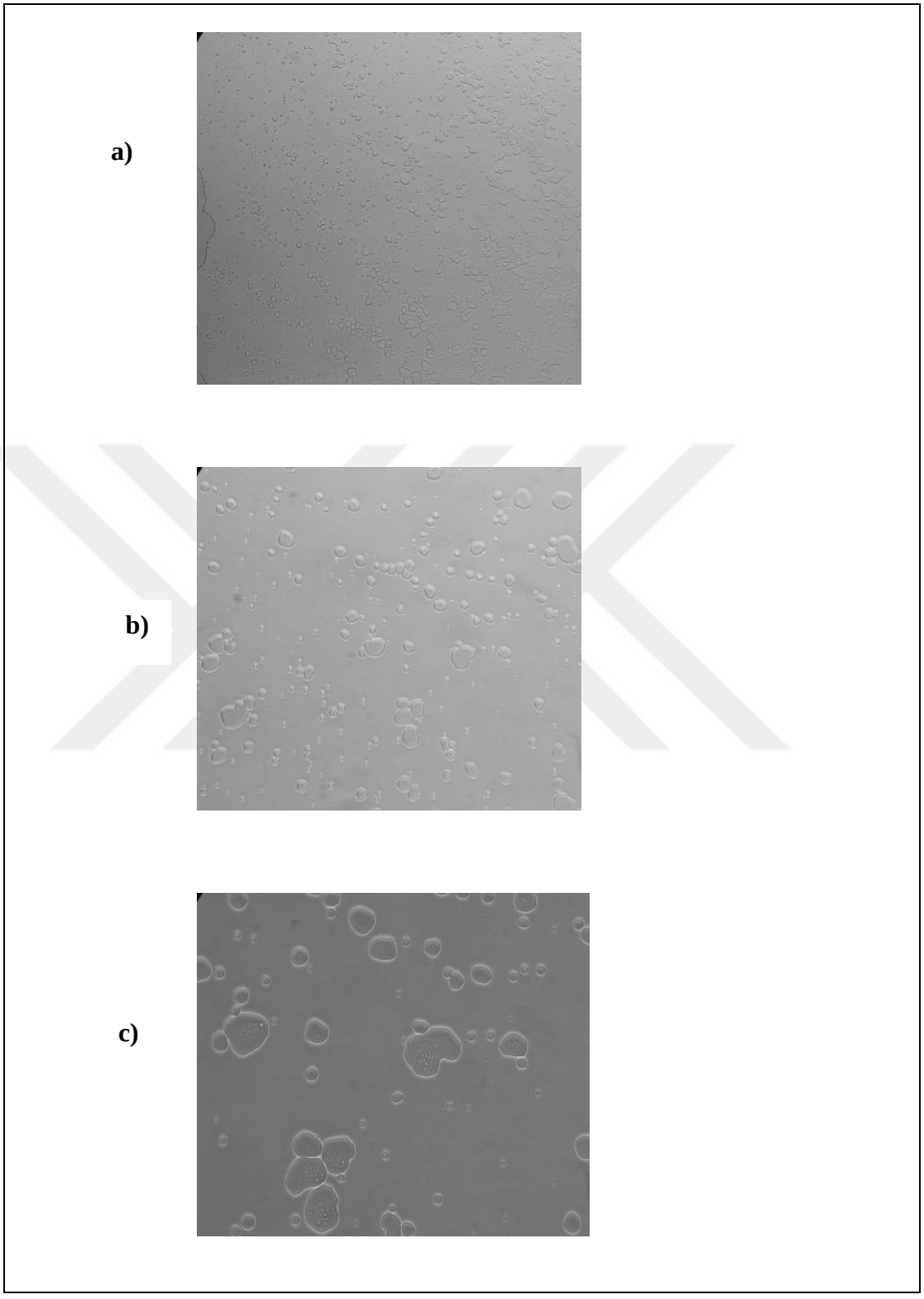

Figure 4.18. Optical micrographs of 2 percent (v/v) Ch including Ch-DW emulsions at a) 10, b) 20, and c) 40X objective magnifications

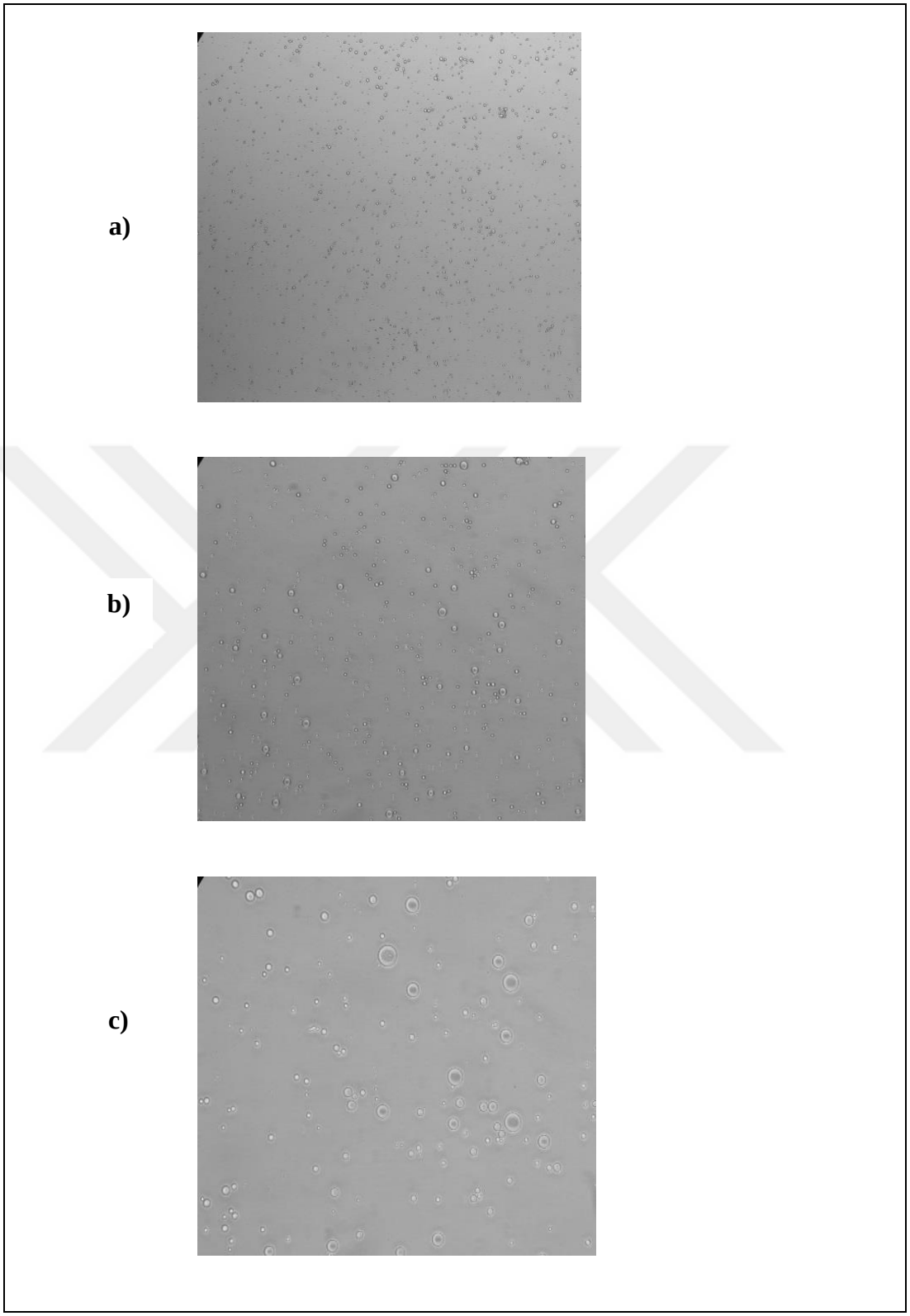

Figure 4.19. Optical micrographs of 2 percent (v/v) Ch including O-DW emulsions at a) 10, b) 20, and c) 40X objective magnifications

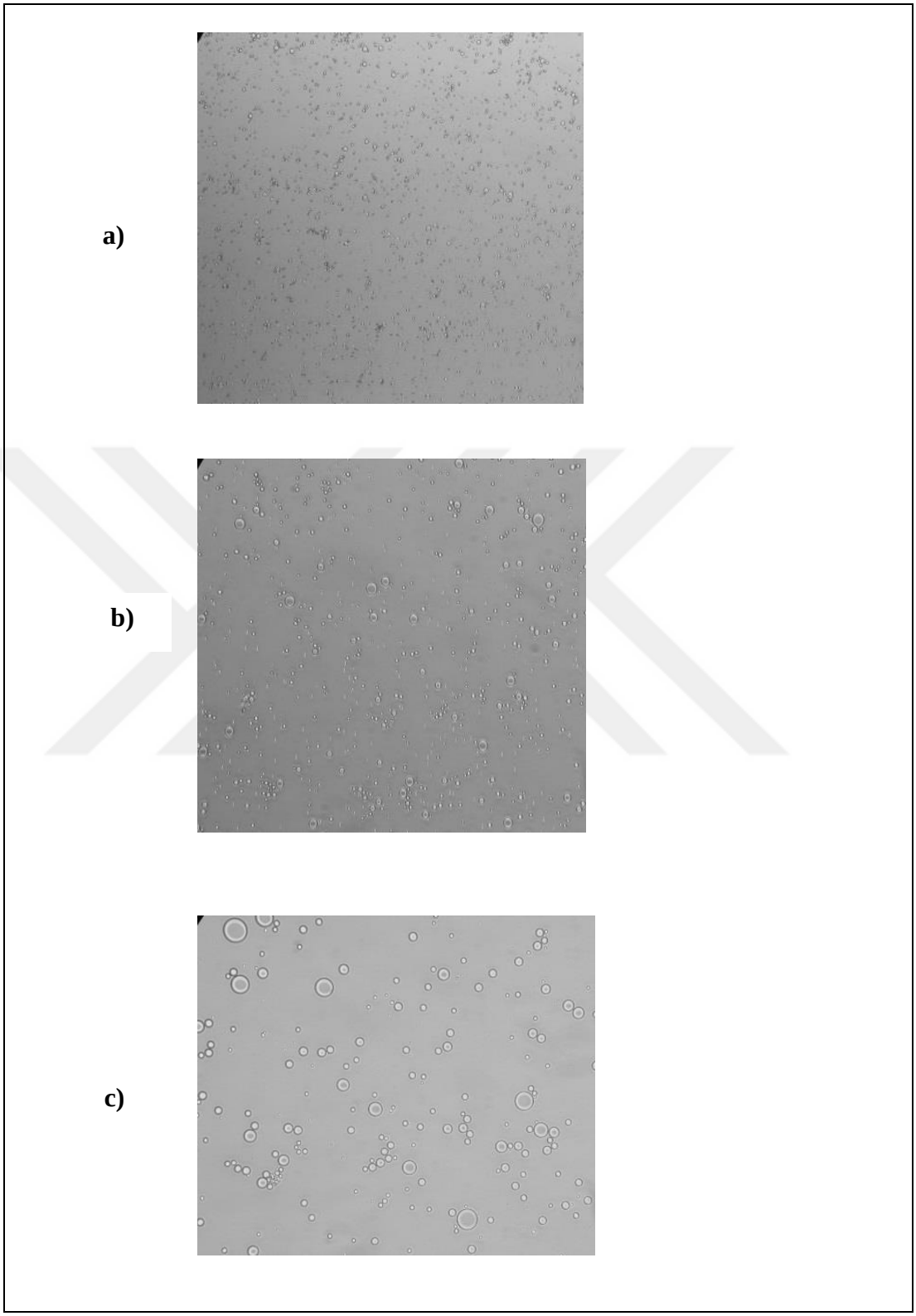

Figure 4.20. Optical micrographs of 2 percent (v/v) Ch including Blk emulsions at a) 10, b) 20, and c) 40X objective magnifications

After the chitosan concentration optimization studies, 1 percent chitosan concentration was chosen for additional ionic strength and pH treatments. For this purpose 1 percent chitosan including emulsions were prepared according to the dropping chitosan in oil, dropping oil in chitosan, and bulk mixing of oil and chitosan solution phases emulsification methods. Treatment applied emulsions were compared against blank samples according 1, 3, 5, 7, and 14 storage time period. Emulsions were evaluated according to mean droplet size, zeta potential, viscosity analyses. The pH 5 effect on the mean droplet size values for C-DW, O-DW and Blk mixing methods were assessed (Figure 4.19).

The response of emulsions to pH 5 in terms of droplet size variation was ranged between 7.9-6.3, 4.1- 9.1, 6.6-9.7 μm for Ch-DW, O-DW, and Blk mixing methods regardless of time effect respectively (Figure 4.21). Also emulsions that pH values were adjusted to 9 exhibited 4.2-9.6, 3.7-7.1, 3.2- 6.7 μm for Ch-DW, O-DW, and Blk mixing methods regardless of time effect respectively (Figure 4.21). When the results compared with blank samples of each method individually, acid and base applied emulsions produced slightly small mean droplet values than their blank samples values and their blank sample results ranged between 5.1-10.0, 4.0-10.9, and 5.0-11.3 μm (Figure 4.23) for Ch-DW, O-DW, and Blk emulsification methods.

On the other hand, the effect of sodium chloride on droplet size of emulsions were evaluated and for Ch-DW emulsification method results were between 3.5-9.6 and 5.4-9.4 μm for 100 and 200 mM NaCl treatment, respectively (Figure 4.22). These results were almost smaller than corresponding blank sample values except 3 and 5. days droplet size values. Emulsions that processed with O-DW method did not show regular behavior against NaCl effect and results ranged between 5.4-9.4, and 5.9-10.6 μm for 100 and 200 mM NaCl, respectively. Also, there were raises and falls in O-DW blank emulsion droplet size values. One hundred and 200 mM NaCl application to Blk mixing emulsified samples caused the droplet size values between 4.7-12.3 and 3.7-7.9 μm respectively. The higher sodium chloride application caused more smaller values and these values were smaller than corresponding blank sample values that were between 11.3-5.0 μm (Figure 4.23).

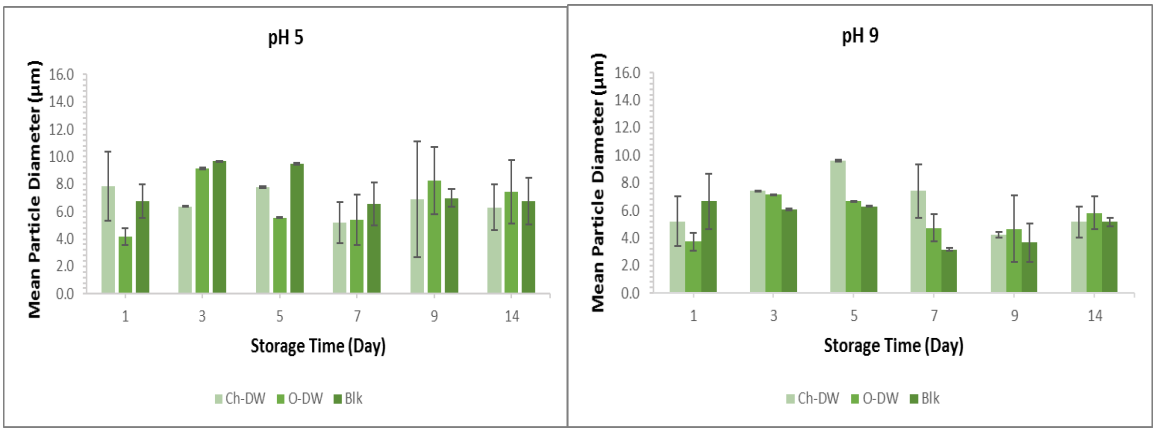

Figure 4.21. Droplet size measurements of 1 percent (v/v) Ch including emulsions at pH a) 5, and b) 9

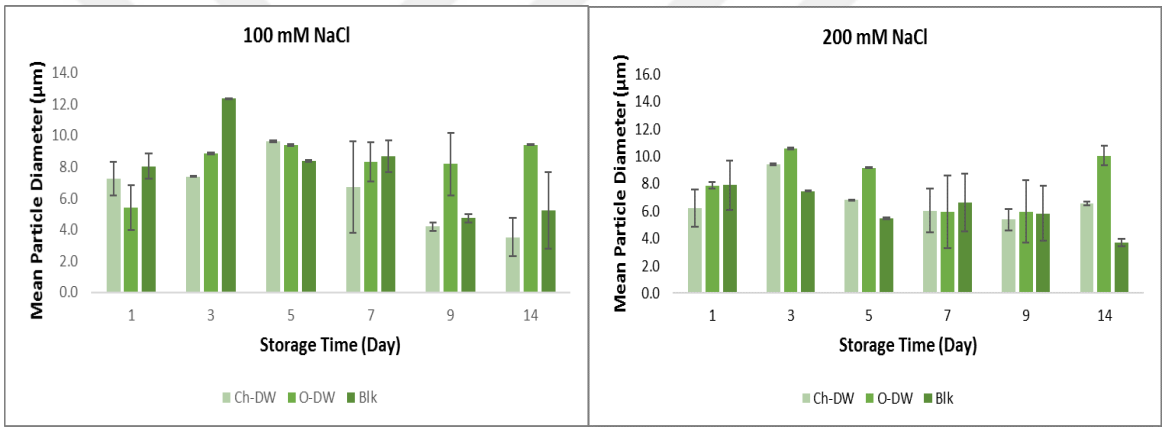

Figure 4.22. Droplet size measurements of 1 percent (v/v) Ch including emulsions at a) 100, and b) 200 mM NaCl

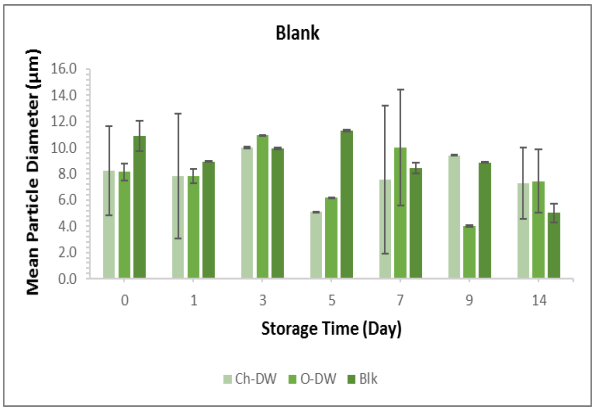

Figure 4.23. Droplet size measurements of 1 percent (v/v) Ch including blank emulsions

Besides droplet size, effect of sodium chloride and acid and base treatment on ζ-potential values of samples were evaluated. ζ-potential values of Ch-DW emulsion blank samples increased from 55.6 to 68.9 mV regardless of time (Figure 4.26). On the other hand, Ch-DW emulsions that pH values adjusted to 5 and 9 showed ζ-potential values between 56.9-64.9 and 54.9-62.1 mV (Figure 4.24) respectively. After first day ζ-potential values of treated samples were smaller than corresponding blank samples. Like Ch-DW emulsion samples, ζ-potential values of O-DW method samples were smaller than their corresponding blank sample values and they were ranged 57.3- 61.6, 54.1-62.1 and 60.1-69.3 mV (Figure 4.24) for pH 5, 9 and blank samples respectively. The highest and smallest ζ-potential values of Blk emulsions without any application were 67.5 and 63.2 mV (Figure 4.24), respectively. Acidic and basic medium caused the variation in ζ-potential of Blk samples as 56.2-63.3 and 50.6-58.4 respectively. There was small decreasing against blank emulsion sample for Blk mixing emulsification method. In terms of method comparison for pH 5 results Ch-DW and Blk mixing methods produced results in the approximate range but for pH 9 results Ch-DW and O-DW exhibited similar ranged results. On the other hand, in general as the sodium chloride concentration increased , ζ-potential values of Ch-DW emulsion were decreased (Figure 4.25) and their values were smaller than corresponding blank sample values, 56.0-61.6, 50.8-57.8 mV for 100 and 200 mM NaCl samples respectively. Like Ch-DW samples, in general O-DW samples produced smaller ζ-potential values than their corresponding blank values. Lastly, ζ-potential values of Blk mixing method were ranged between 47.4-59.1 and 49.9-57.6 mV for 100 and 200 mM NaCl concentrations. As seen from Figure 4.25 for 100 mM NaCl concentration Ch-DW and O-DW showed similar ranged ζ-potential values which were higher than Blk mixing method. But in terms of method comparison for high chloride concentration, it was not possible to sort the methods.

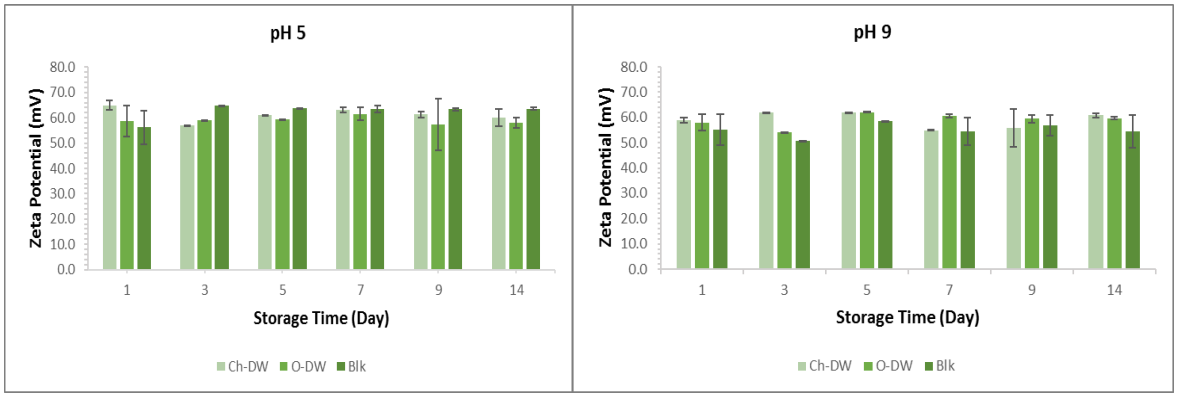

Figure 4.24. Zeta potential measurements of 1 percent (v/v) Ch including emulsions at pH a) 5, and b) 9

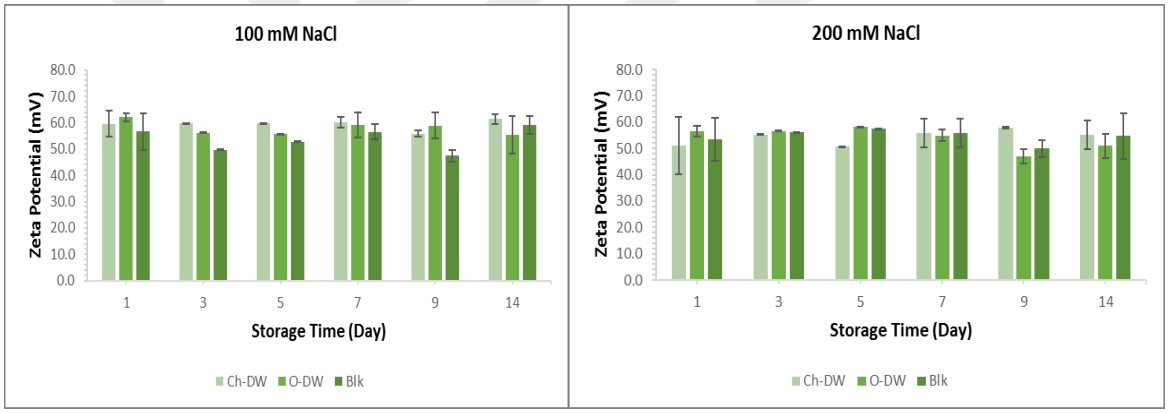

Figure 4.25. Zeta potential measurements of 1 percent (v/v) Ch including emulsions at a) 100, and b) 200 mM NaCl

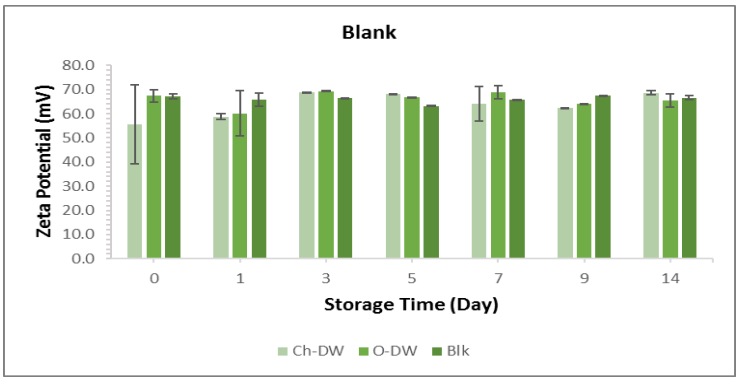

Figure 4.26. Zeta potential measurements of 1 percent (v/v) Ch including blank emulsions

4.1.6. Modification Degree of Chitosan-*g*-Caffeic Acid

Chitosan-g-caffeic acid complexes were assed to find best caffeic acid binding ratio. Binding ratio results of the samples were 0.8, 4.3, and 8.2 percent for 5, 10, 20 mM caffeic acid concentrations, respectively. Therefore, 20 mM Ch-g-Ca sample were chosen due to its higher caffeic acid ratio for further emulsion studies.

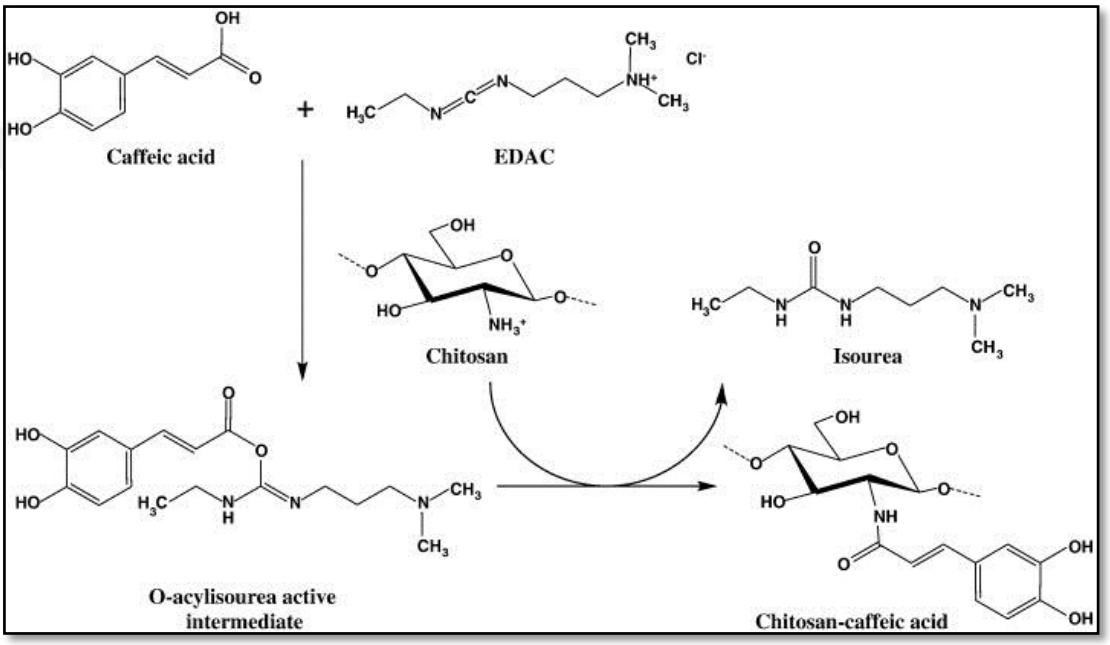

Figure 4.27. Grafting reaction of caffeic acid on chitosan [17]

4. 1.7. Measurement Antioxidant Activity of Chitosan-*g*- Caffeic Acid

Antioxidant activity of chitosan-*g*-caffeic acid complexes were evaluated by radical-scavenging activity and reducing power. Radical scavenging activity is based on reducing ability of DPPH which is free radical by accepting an electron or hydrogen to be stable [17]. Results of the radical scavenging activity were defined as half-inhibition concentration (IC_{50}) and they were 2.5, 1.3, 0.6 mg/ml (Figure 4.27) for 5, 10, 20 mM caffeic acid containing chitosan-*g*-caffeic acid complexes respectively. There was a directly proportional correlation between caffeic acid concentration and IC_{50} amounts of

samples. It means that the highest radical scavenging activity showing sample was the 20 mM caffeic acid containing sample. Besides radical scavenging activity, reducing power outcomes were evaluated. Reducing power is related reduction of Fe^{3+}/ferricyanide complex to the ferrous form in the presence of antioxidant agents [17]. The results were expressed as effective concentration (EC_{50}) and they were 1.8, 0.3, 0.4 mg/ml (Figure 4.28) for 5, 10, 20 mM caffeic acid containing chitosan-*g*-caffeic acid complexes respectively. Similar IC_{50} results, lowest EC_{50} value was obtained for lowest caffeic acid containing complex, but the EC_{50} values of the 10 and 20 mM caffeic acid including complexes presented slightly similar results.

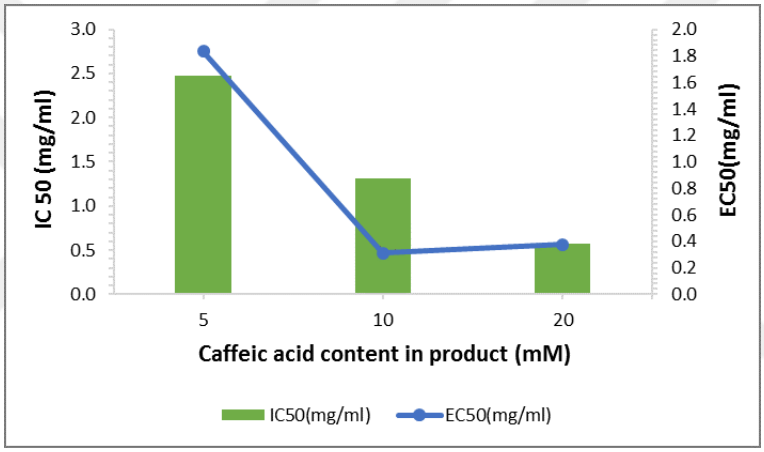

Figure 4.28. Half-inhibition concentration (IC_{50}) of radical scavenging activity and effective concentration 50 (EC_{50}) of reducing power measurement of chitosan-*g*-caffeic acid complexes with different caffeic acid content

4.1.8. Emulsion Preparation using Chitosan-*g*- Caffeic Acid

After the 1 percent chitosan including emulsion preparation same procedure was applied for 1 percent chitosan-*g*-caffeic acid complex including emulsions. However there was no decision for most effective emulsification method, Ch-*g*-Ca-DW, O-DW, and Blk mixing methods were examined again for 1 percent chitosan-*g*-caffeic acid including emulsion process and samples were evaluated against 1, 7, and 14 day storage duration. Effect of acidic environment on droplet size values of emulsion were examined and the lowest and

highest droplet values of Ch-*g*-Ca-DW, O-DW (Figure 4.29) ,and Blk mixing methods were 4.9, 4.1,5.1 µm and 6.0, 7.5, 6.3 µm respectively. Each method produced droplet values smaller than corresponding blank values, . The results of the basic environment for O-DW and Blk mixing methods were irregular and there was not consistency with each other and blank samples. On the other hand, Ch-*g*-Ca-DW emulsified samples produced approximate droplet size values to pH 5 results. Addition to droplet values, impact of acidic and basic environment on ζ-potential values were assessed. Acidic environment caused to reduction of ζ-potential values of Blk emulsion against blank samples and followed a decreasing trend from 24.2 to 22.2 mV (Figure 4.32). Similarly the ζ-potential values of Ch-*g*-Ca-DW samples exhibited tendency to reduction as 27.4-21.8 mV (Figure 4.32). O-DW samples showed some rises and falls during whole storage period and higher values their blank samples (Figure 4.32). Ch-g-Ca-DW and Blk methods showed approximate lowest and highest value regardless of time, both method produced 22.3 mV as lowest ζ-potential value and 25.0 and 25.1 mV as highest values respectively. Moreover, O-DW method the highest and lowest values were 20.5 mV and 24.6 mV respectively. Also there were not increasing or decreasing for three method related with time but in general they were smaller than blank values.

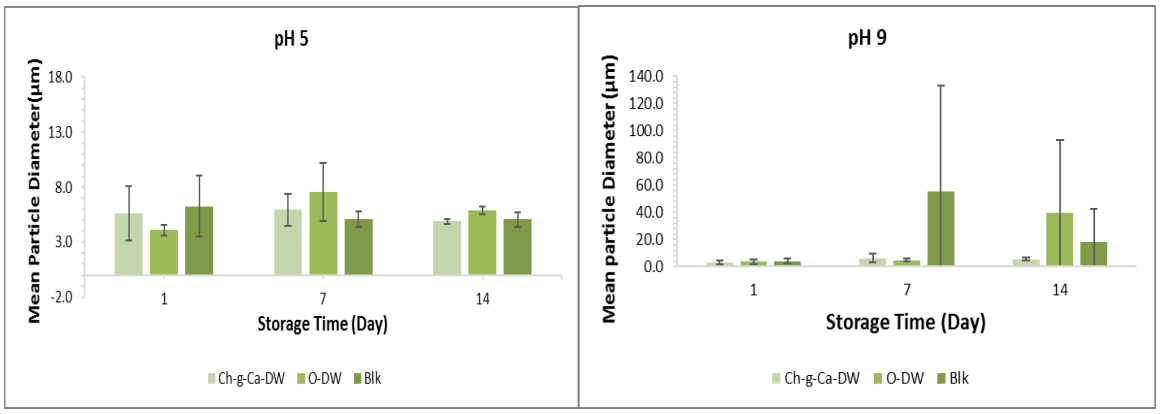

Figure 4.29. Droplet size measurements of 1 percent (v/v) Ch-*g*-Ca including emulsions at pH a) 5, and b) 9

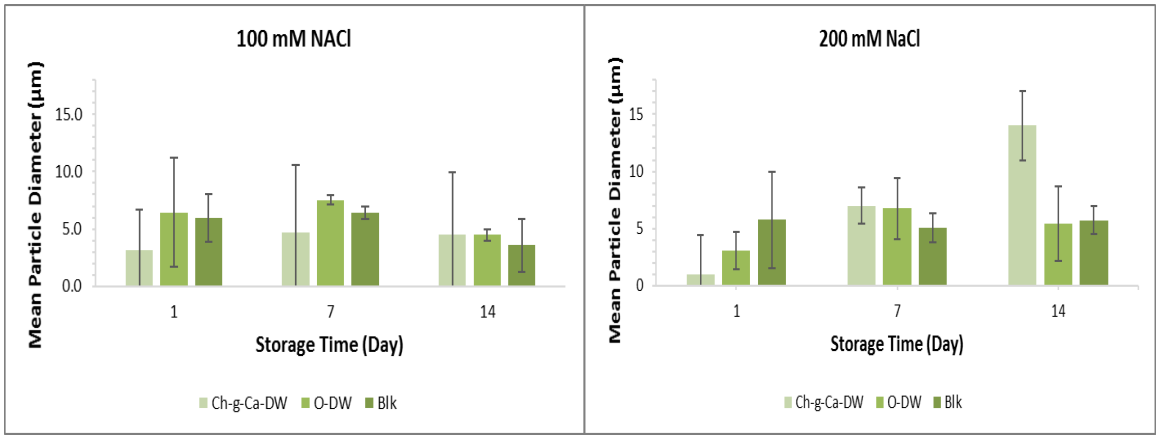

Figure 4.30. Droplet size measurements of 1 percent (v/v) Ch-*g*-Ca including emulsions at a) 100, and b) 200 mM NaCl

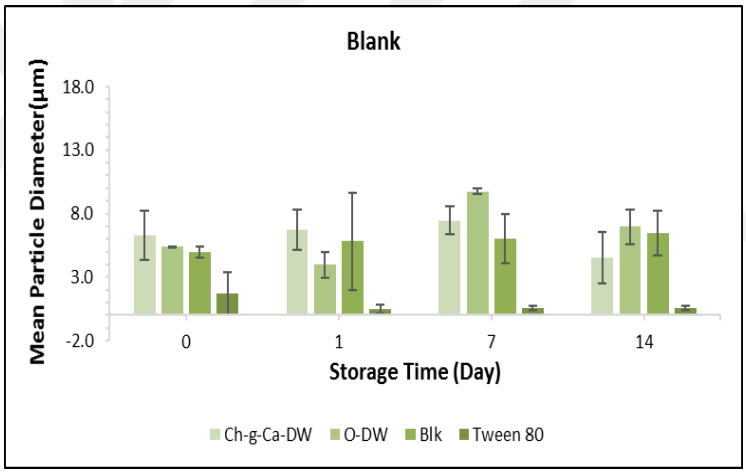

Figure 4.31. Droplet size measurements of 1 percent (v/v) Ch-*g*-Ca including blank emulsions

Sodium chloride influence were examined aspect of both droplet size and ζ-potential value for all three method. In terms of droplet size measurements, though all three method did not present a consistent relation with time, in general their values were smaller than corresponding blank sample values. The lowest and highest values for 100 mM NaCl treatment were 3.1, 4.5, 3.6 µm and 4.7, 7.5, 6.4 µm for Ch-*g*-Ca-DW, O-DW, and Blk mixing methods respectively (Figure 4.30). Also these values for 200 mM NaCl process were 5.2, 3.1, 5.0 µm and 6.3, 6.8 and 5.8 µm for Ch-*g*-Ca-DW, O-DW, and Blk mixing methods respectively. The concentration relation could be observed for Ch-*g*-Ca-DW

method, as the concentration increased droplet values were decreased (Figure 4.30). In the case of sodium chloride influence on ζ-potential values, in general there were rises and falls in the values of all three method samples and all results were smaller than corresponding blank sample result. The Ch-*g*-Ca-DW method caused the higher ζ-potential values for both concentration than other two method, 26.6 mV and 25.0 mV (Figure 4.33) for 100 and 200 mM NaCl concentrations respectively but there were not a certain concentration effect. The higher sodium chloride cause the relatively smaller ζ-potential values for O-DW method samples, the highest values were 23.0 and 21.7 mV (Figure 4.33) for 100 and 200 mM NaCl concentrations. On contrast to O-DW method, as the concentration increased the value of ζ-potential was increased and the highest values were 22.6 and 24.5 mV (Figure 4.33) for lower and higher sodium chloride concentrations respectively. Also, 0.5 (w/v) % Tween 80 (according the codex value) stabilized emulsion sample were compared with 1 percent chitosan-*g*-caffeic acid, and it lead to production of droplets with 0.5-1.7 μm range and with -13.6-10.2 mV ranged ζ-potential values.

When the ζ-potential and droplet size values of 1 percent chitosan and 1 percent chitosan-*g*- caffeic acid compared with each other, droplet size values of 1 percent chitosan blank emulsions were smaller than corresponding 1 percent chitosan-*g*-caffeic acid containing blank emulsions for all three methods (Figure 4.23, 4.31). Additionally, the presence of caffeic acid caused the reduction of ζ-potential values of all three methods both blank emulsion samples and all treated ones. (Figure 4.34).

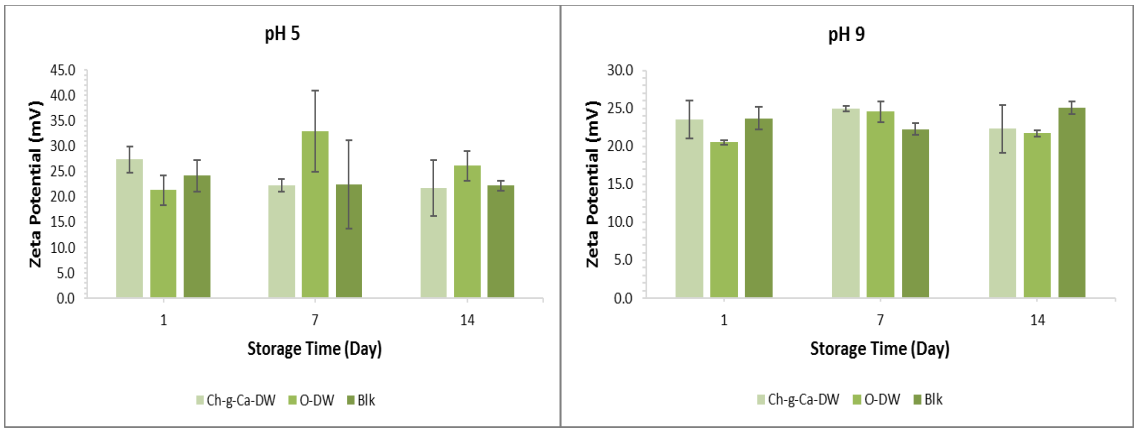

Figure 4.32. Zeta potential measurements of 1 percent (v/v) Ch-*g*-Ca including emulsions at pH a) 5, and b) 9

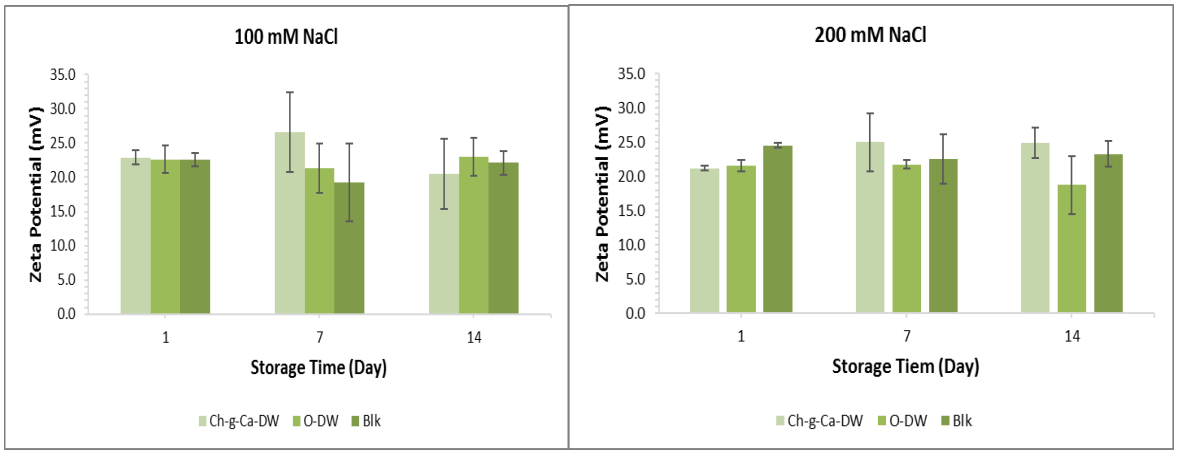

Figure 4.33. Zeta potential measurements of 1 percent (v/v) Ch-*g*-Ca including emulsions at a) 100, and b) 200 mM NaCl

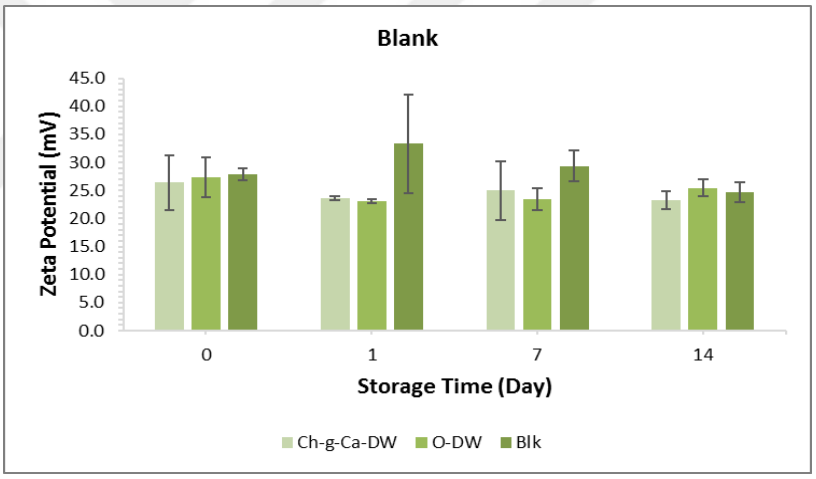

Figure 4.34. Zeta potential measurements of 1 percent (v/v) Ch-*g*-Ca including blank emulsions

4.1.9. Creaming Stability and Microscope Observation of Chitosan-*g*- Caffeic Acid Emulsion

The creaming phase formation of 1 percent chitosan-*g*-caffeic acid containing emulsion was measured at 1, 7, and 14. storage days by visual observation (Figure 4.36). Emulsion creaming formation was fixed at the end of first day for all three method and results were

23.9, 23.4, and 21.7 % for Ch-*g*-Ca-DW, O-DW, and Blk mixing methods respectively. Additionally, creaming rate values were showed approximate values as seen Figure 4.35. The evaluation of creaming fraction values of 1 percent chitosan-*g*-caffeic acid containing emulsions revealed that these results were higher than corresponding values of 1 percent chitosan containing emulsions for all three methods (Figure 4.35) Microscope observation indicated that production of small spherical shape droplet formation for both Ch-*g*-Ca-DW and Blk methods than O-DW method which had larger droplets somewhere. There was no clear indication of aggregation of droplets (Figure 4.37).

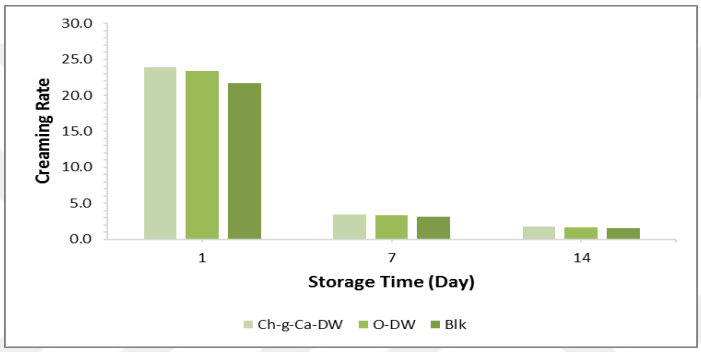

Figure 4.35. Creaming rate of 1 percent (v/v) Ch-*g*-Ca including emulsions

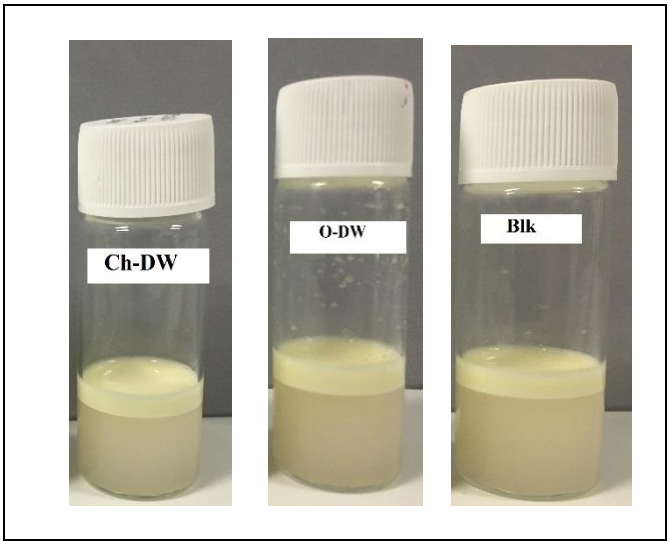

Figure 4.36. Visual appearance of 1 percent (v/v) Ch-*g*-Ca including Ch-*g*-Ca-DW, O-DW, and Blk emulsions during storage

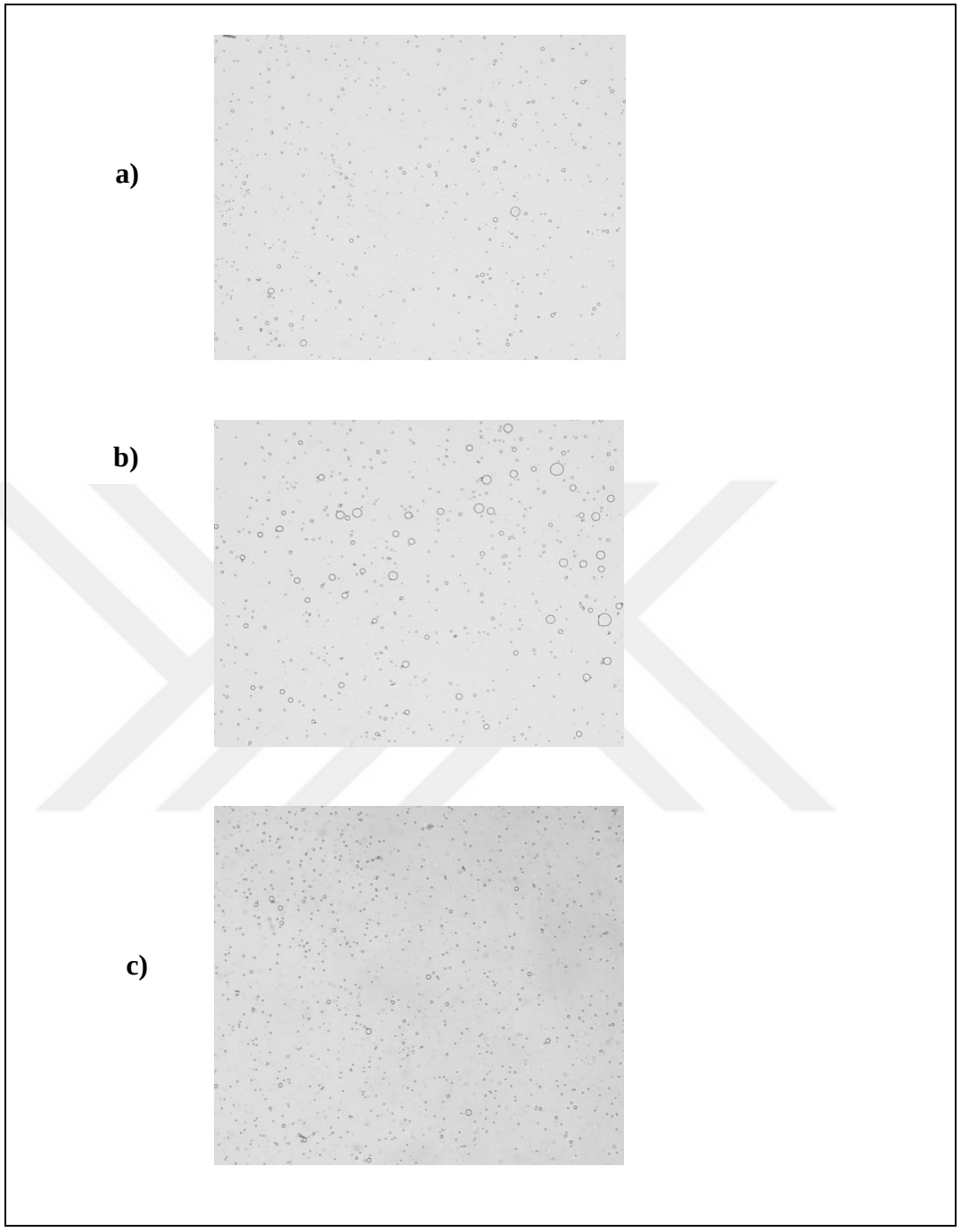

Figure 4.37. Optical micrographs of 1 percent (v/v) Ch-*g*-Ca including a) Ch-*g*-Ca-DW b) O-DW, and c) Blk emulsions at 5X objective magnification

4.1.10. Antioxidant Activity of Chitosan-*g*- Caffeic Acid Emulsion

The antioxidant values of emulsions produced from 1 percent chitosan-*g*-caffeic acid complex via three different emulsification methods were evaluated at 1. and 14. days after

the formation. The results of radical scavenging activity measurement revealed that (Table 4.1) although both three method exhibited radical scavenging activity, these results decreased at the end of the storage period regardless of the type of the emulsification method. At the initial of storage period antioxidant activity followed a decreasing trend from Ch-*g*-Ca-DW method to Blk mixing methods, and results were 21.0, 14.4, and 4.9 percent respectively. Also radical scavenging results were smaller than activity of 20 mM chitosan-*g*-caffeic acid complex which was average 48.6 percent. Unlike radical scavenging activity results, reducing power results displayed a correlation with time. As the storage time increased, reducing power values showed slightly raise (Table 4.2). The results of the all three method for first day were the same, and it was 1.4 absorbance value at 700 nm. However at the end of the whole storage duration, reducing power activity of samples exhibited slightly increasing trend and values were 1.7, 1.8, and 1.9 absorbance value at 700 nm. When these results compared reducing power value of 20 mM chitosan-*g*-caffeic acid, average 0.5, it was revealed that emulsion values were higher than it.

Table 4.1. DPPH activity of 1 percent (v/v) Ch-*g*-Ca including emulsions

DPPH Activity (%)		
Emulsification Method/ Storage Time (Day)	**1**	**14**
Ch-g-Ca-DW	21.0 ±0.75	-6.08±14.28
O-DW	14.4 ±32.09	-90.21±74.19
Blk	4.9±23.22	-62.90±34.43

Table 4.2. Reducing power activity of 1 percent (v/v) Ch-*g*-Ca including emulsions

Reducing Power @700 nm Abs		
Emulsification Method/ Storage Time(day)	**1**	**14**
Ch-g-Ca-DW	1.4±0.09	1.7±0.09
O-DW	1.4±0.01	1.8±0.15
Blk	1.4±0.02	1.9±0.10

4.1.11. Viscosity of Emulsions

Viscosity measurements of the emulsions that composed of 1 percent chitosan and 1 percent chitosan-*g*-caffeic acid complex for all three method were evaluated against the same percent chitosan and chitosan-*g*-caffeic acid complex which had viscosity values as

126.7 and 48.6 cP respectively. As seen Figure 4.38 viscosity results of 1 percent chitosan including emulsions were smaller than the same concentration 1 percent chitosan-g-caffeic acid complex including emulsions regardless of emulsification method. The viscosity values of emulsions that made up chitosan-*g*-caffeic acid presented 65.3, 67.2, and 49.9 cP values for Ch-*g*-Ca-DW, O-DW, and Blk mixing methods respectively and these values were approximate to viscosity of chitosan-*g*-caffeic acid complex, 48.6 cP. The comparison of methods exhibited that viscosity of Blk mixing method smaller than other two methods which had slightly similar viscosity results. On the other hand, viscosity results of emulsions produced from 1 percent chitosan from lowest to highest were 95.4, 107.5, and 138.2 cP for O-DW, Blk mixing and Ch-DW methods respectively. The Ch-DW method produced approximate result to viscosity of 1 percent chitosan, 126.7 cP.

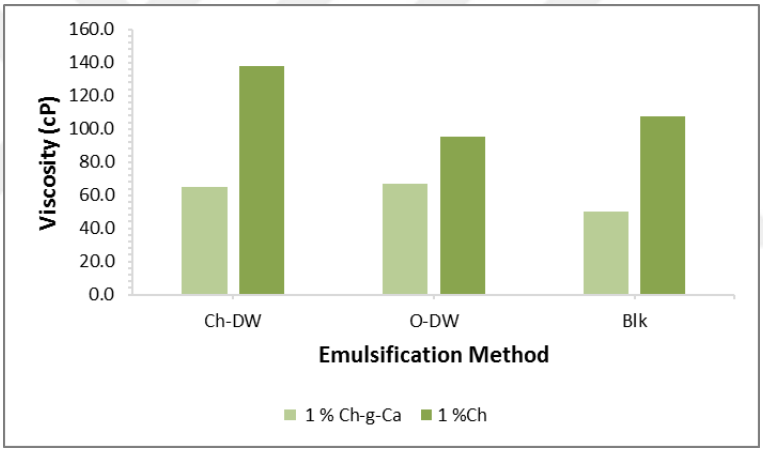

Figure 4.38. Viscosity measurements of 1 percent (v/v) Ch, a
and Ch-*g*-Ca including emulsions

5.DISCUSSION

Chitosan is positively charged polysaccharide and it has pKa value around 6.3-7 at neutral and acidic environment [15, 27]. Chitosan is made up from β-(1-4)-linked 2 -amino-2-deoxy-D-glucose and poly *N*- acetyl-D-glucosamine parts which give it hydrophilic and hydrophobic nature, respectively [24]. Therefore chitosan can behave like surfactants with distinct hydrophile- lipophile balance (HLB) values [25]. In the literature there have been numerous studies that deal with emulsifier or stabilizing activity of using chitosan in oil in water (o/w) emulsions. Some of these studies are based on emulsification of o/w emulsion by only chitosan [15, 24, 25, 37, 38] , some of them include combination of chitosan with other polysaccharides and other surfactants [26-30, 33, 34, 39-44]. There are limited studies based on chitosan modification to investigate its emulsifier or stabilizer activity [45]. Even there are limited studies based on emulsifier or stabilizing activity of chitosan that modified by introducing polyphenol compounds into the backbone of it [18, 20-23]. This study made up from four basic parts which are initially optimization homogenization and chitosan concentration, determination most effective chitosan concentration for all three homogenization method, modification of chitosan by caffeic acid and including binding ratio and antioxidant measurements of modified chitosan complex, lastly emulsion preparation from that Ch-*g*- Ca complex and comparison its results with only Ch including emulsion characterization results.

In first part, emulsifier activity of chitosan was tested with different homogenization techniques such as homogenization with high shear blender, ultrasonication, thermal treatment and combination of them with arrangement of different process conditions such as speed of the high shear blender, temperature, and duration of treatment. When the droplet size results were evaluated for first three optimization studies, 1 min sonication, 5 min sonication and combination of homogenization with high shear blender at 25, 000 rpm for 5 min, and heating all ingredients up to 65 ℃ in water bath and addition 5 min sonication process were produced best results in each involved method. Although sonication method lead to formation of small droplets most of it energy dissipated as heat so this method was eliminated. At the end of the optimization studies dropping method under heating condition was chosen to understand adsorption capability of chitosan and

then homogenization with high shear blender was added to obtain complete emulsion system.

The Ch concentration optimization studies exhibited that for 2 percent (v/v) Ch based emulsion produced higher droplet size values, also microscope observation was supported these higher droplet size results especially for Ch-DW method droplets were not in spherical shape. On the other hand 1 and 1.5 percent (v/v) Ch based emulsions produced similar value for Ch-DW method but there was not a consistent for O-DW and Blk methods for two concentrations. But 1.5 percent (v/v) Ch concentration lead to slightly higher creaming fraction values and microscope images revealed that there were some aggregation in this emulsion type. The reason of higher droplet size values of 2 percent (v/v) Ch based emulsion and aggregation observation for 1.5 and 2 percent (v/v) Ch might be associated with non- adsorbed chitosan present in the continuous phase [46]. Also as the Ch concentration increased, mean droplet size values increased for Ch-DW and O-DW methods and this case might be attributed to slow flow rate due to higher chitosan concentration for Ch-DW method and slow or insufficient mixing of chitosan for both two method. Therefore, in both cases decreasing in speed of adsorption of chitosan molecules by oil droplet surfaces could lead to higher droplet size values. On the other hand, for Blk emulsification method 1.5 percent Ch presented lowest value and 1 percent Ch highest value. There might be different approaches for this outcome such as lowest Ch concentration is not sufficient to cover the droplet surfaces or it is not related with concentration effect, it could be explained with operation conditions such as mixing speed. Aggregation formation in 1.5 and 2 percent (v/v) chitosan based samples also might be explained that addition of chitosan lead to increase in depletion attraction between droplets and when attraction forces overcome repulsive forces droplets flocculated [46]. Therefore in Ch-DW type emulsion slow addition of chitosan could lead to examination of this flocculation more clear. The reasons of droplet flocculation might be insufficient adsorption of chitosan by oil droplets that chitosan was not able to cover completely the droplet surface thereby cause to bridging flocculation. The chitosan concentration effect was not similar to study conducted investigation stabilizing activity of chitosan with the presence of whey protein isolate [27]. It was suggested that increasing Ch concentration lead to decreasing in average droplet size but in this study increasing in concentration lead to large droplet formation. However creaming stability results might be similar because in higher concentration creaming layer observation was late because of the increasing in

viscosity. In the literature it was proposed that creaming rate value less than 1 mm/day can be assumed as stable against creaming [11]. Although, in this study creaming rate was calculated as creaming fraction per day, the cream layer thickness were less than 1 mm.

At the pH 9 value of 1 percent Ch and Ch-*g*-Ca including emulsions coagulation was observed this might be associated that as the pH value of the emulsion increases association between chitosan and water decreases [15] and also in acidic medium chitosan has positive charge because of its protonated amino groups (pK_a ~6.3-7.0) thereby pH values higher than 6.5 causes insolubility problems of chitosan [46]. The zeta potential is an critical value that related with stability of emulsion. When the ζ-potential values of 1 percent Ch including emulsion droplets compared although there was decreasing for all samples against blank sample but it was obvious for NaCl treated ones, this might be associated with electrostatic screening effect of NaCl. The viscosity measurement results of 1 percent (v/v) Ch and 1 percent (v/v) Ch-*g*-Ca containing emulsions presented that without caffeic acid emulsions had higher viscosity value. It was suggested that by some researches the stabilizing activity of chitosan is contributed to its viscosity increasing ability of aqueous phase [15, 24, 25, 46]. Also blank sample comparison of 1 percent (v/v) Ch and 1 percent Ch-*g*-Ca showed that in the presence of with caffeic acid emulsions produced more smaller droplets. These two outcome might be associated that rapid creaming layer formation so higher creaming fraction values of Ch-*g*-Ca samples, it was suggested that the raise in viscosity of aqueous phase can decrease creaming rate [46]. The low viscosity of caffeic acid modified emulsion might be related with rapid solubility of modified chitosan sample against native chitosan. Moreover, zeta potential value of emulsion with caffeic acid modified samples were smaller than only chitosan based emulsions and this might be a reason instability of emulsions because low zeta potential value can be reason of weak electrostatic repulsive forces [46]. This situation might be caused by lower zeta potential value of Ch-*g*-Ca (~9 mV) than chitosan including emulsions that lead to decreasing in surface charge of emulsions.

The radical scavenging activity results of caffeic acid modified chitosan including emulsions showed an decreasing in antioxidant activity this might be contributed to poor solubility of caffeic acid in oil, location in the aqueous phase [21]. With caffeic acid modification of chitosan there might be blocking of amine groups that cause the aggregation of chitosan thereby prevent it meet with solvent in emulsion. This situation can prevent showing antioxidant capability of caffeic acid. The decreasing in radical

scavenging activity of Ch-*g*-Ca based emulsions during storage might be deal with sensitivity of caffeic acid to storage conditions such as light and temperature.

As a result, the inconsistency between emulsification methods might be related with distinct flow rate and dropping time of oil or chitosan solution. The most efficient system such as adjustable an automatic system might improve emulsification methods to get reasonable results. Moreover, chitosan is made up from molecules with different deacetylation degrees [24, 26] and some variations in oil nature can be observed. Therefore these might be reasons of descent and ascent in droplet size and zeta potential values of emulsions. To make reliable comparison against literature results such parameters should be the same for instance concentration of emulsifier, presence or absence of another emulsifier agent, emulsification method and time but it is difficult to find exactly the same conditions at literature. Therefore comparison of results was made predominantly based on emulsifier type than other parameters. The droplet size value assessment of only chitosan including emulsions were done with literature results [15, 24, 25] and it was noticed that droplet size values raised to 100 μm and they were bigger than droplet size values of 1 percent (v/v) Ch including emulsions. On the other hand, in comparison to modified chitosan emulsion studies most droplet size results were in nanoscale range in which was used high efficiency homogenization machine, but with chitosan tripolyphosphate [23] nanoparticles used in pickering emulsion produced droplet size at least ~ 40 μm which are extremely higher than Ch-*g*-Ca results.

In the literature, stabilizing and emulsifying agents are considered as different agents due to functionality in somewhere [2] and polysaccharides are assumed as an stabilizing agents that can act in the presence of a surfactant or other agents to improve texture and creaming stability by increasing viscosity [27] Although there is need of improvements and there are some variabilities between results of the study, it was shown that both chitosan and chitosan-*g*-caffeic acid complex had ability to produce emulsions and can be stabile during storage in the absence of a surfactant agent.

6. CONCLUSION

In this study, oil in water emulsion was formed with different emulsification techniques by using extra virgin olive as oil phase and chitosan and chitosan-*g* caffeic acid derivative as emulsifier agents. Different emulsification techniques, Ch-DW, O-DW, and Blk, were improved to reduce negative effect of insolubility of chitosan during absorption to the oil droplets. 1 percent (v/v) chitosan solution value was chosen to obtain an economically emulsion. The results of the study showed that both chitosan and chitosan-*g*-caffeic acid can produce emulsion with enough stability. Although modification of Ch-*g*-Ca had antioxidant activity, it did not transfer this property to the emulsion sufficiently. Therefore with the improvements in emulsification process, amount of caffeic acid in chitosan derivative , and solubility of Ch-*g*-Ca in oil, it is possible to improve emulsifying, and stabilizing activity and antioxidant property of Ch-*g*-Ca in oil in water emulsions. Additionally, the emulsion system can be candidate for encapsulation method of compounds such as flavors, colors, micronutrients, antimicrobials in food and pharmaceutical industries.

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